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(57) Abstract			
<p>The present invention relates to a novel method for the production of short chain polypeptides, including polypeptides having up to 3 disulfide bonds and/or structures rich in basic amino acid residues, and open structured short chain polypeptides, e.g. glucagon, glucagon like peptides and their functional analogues, in genetically modified yeast cells having reduced activity of YAP3 protease. The invention further comprises genetically modified yeast cells, and a method for the preparation of said yeast cells.</p>			

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METHOD FOR THE PRODUCTION OF POLYPEPTIDES

FIELD OF THIS INVENTION

5 The present invention relates to a novel method for the production of short chain polypeptides, including polypeptides having up to 3 disulfide bonds and/or structures rich in basic amino acid residues, and open structured short chain polypeptides, e.g. glucagon, glucagon like peptides and their functional analogues, in genetically modified yeast cells, said genetically modified yeast cells, and a method for the
10 preparation of said yeast cells.

BACKGROUND OF THIS INVENTION

15 Expression of heterologous proteins in yeast after transformation of yeast cells with suitable expression vectors comprising DNA sequences coding for said proteins has been successful for many species of polypeptides, such as glucagon, glucagon like peptides and their functional analogues. Yeasts, and especially *Saccharomyces cerevisiae*, are preferred host microorganisms for the production of pharmaceutically
20 valuable polypeptides due to the stable yield and safety.

However, it is often found that the expression product is a heterogeneous mixture of species of the desired polypeptide precursor having different amino acid chain lengths. A number of proteases, activated by the PEP4 gene product are responsible for yeast
25 protein degradation. Mutation in the PEP4 gene such as the pep4-3 mutation is often used to reduce cellular proteolysis whereby the quality and yields of heterologous proteins of interest can be improved. EP 341215 describes the use of a yeast strain that lacks carboxypeptidase ysc α activity for the expression of the heterologous protein hirudin. Wild-type yeast strains produce a mixture of desulphatohirudin species
30 differing in the C-terminal sequence due to the post-translational action of endogeneous yeast proteases on the primary expression product. It is shown that

yeast mutant strains lacking carboxypeptidase $ysc\alpha$ activity are unable to remove amino acids from the C-terminus of heterologous proteins and therefore give rise to integral proteins.

The use of yeast strains defective in protease A, B, Y, and/or S activity can only
5 partially reduce random proteolysis of foreign gene products.

Another problem encountered in production of heterologous proteins in yeast is low yield, presumably due to proteolytic processing both in intracellular compartments and at the plasma membrane caused by aberrant processing at internal sites in the protein
10 e.g. secretion of human parathyroid hormone (Gabrielsen et al. Gene 90: 255-262, 1990; Rokkones et al. J. Biotechnol. 33: 293-306, 1994), and secretion of β -endorphine by *S. cerevisiae* (Bitter et al. Proc. Natl. Acad. Sci. USA 81: 5330-5334, 1984). Some polypeptides, e.g. polypeptides having from about 10 to about 55 amino acids or shorter chains and none or a few disulphide bonds and/or are rich in basic
15 amino acids, such as β -endorphine, glucagon and glucagon like peptides may be especially susceptible to intracellular and extracellular proteolytic degradation when expressed in a heterologous host due to their short-chain open and non-stable structure resulting in an inhomogeneous product.

20 WO 95/23857 discloses production of recombinant human albumin (rHA), which is a very large carrier-type protein cross-linked with 17 disulphide bonds and having a molecular weight of about 66 kD, in yeast cells having a reduced level of yeast aspartyl protease 3 (Yap3p) proteolytic activity resulting in a reduction of undesired 45 kD rHA fragment and in a 30 to 50% increased yield of recovered rHA produced by the haploid
25 $\Delta yap3$ yeast strain compared to the rHA produced by the corresponding haploid YAP3 wild-type yeast strain.

Previously, Bourbonnais et al. (Biochimie 76: 226-233, 1994), have shown that the YAP3 protease gene product has in vitro substrate specificity which is distinct though
30 overlapping with the Kex2p substrate specificity, and shown that Yap3p cleaves exclusively C-terminal to arginine residues present in the prosomatostatin's putative

processing sites. Moreover, Cawley et al. (J. Biol. Chem. 271: 4168-4176, 1996) have determined the in vitro specificity and relative efficiency of cleavage of mono- and paired-basic residue processing sites by Yap3p for a number of prohormone substrates, such as bovine proinsulin.

5

The purpose of the present invention is to provide an improved method for the production of secreted polypeptides having up to about 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40 or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no
10 more than one disulphide bond, in the structure in a yeast expression system. Preferred examples of polypeptides are glucagon and glucagon like peptides, CRF, and truncated and/or C-or N-terminally truncated and/or N-terminally extended forms of cocaine amphetamine regulated transcript (CART). Preferably, the production of polypeptides according to the invention is considerably increased, e. g. more than two
15 fold compared to the production of said polypeptides in conventional yeast expression systems.

Often it is advantageous to produce heterologous polypeptides in a diploid yeast culture, because possible genetical defects may become phenotypically expressed in a
20 haploid yeast culture, e.g. during continuous fermentation in production scale, and because the yield may be higher (Fu et al. Biotechnol. Prog., 12: 145-148, 1996; Mead et al. Biotechnol. Letters, 8: 391-396, 1986).

It would be obvious for a person skilled in the art to use the method of the present
25 invention to produce other polypeptides satisfying the above criteria, such as insulin and insulin analogues, adrenocorticotrophic hormones, angiotensinogen, atrial natriuretic peptides, dynorphin, endorphines, galanin, gastrin, gastrin releasing peptides, neuropeptide Y fragments, pancreastatin, pancreatic polypeptides, secretin, vasoactive intestinal peptide, growth hormone releasing factor, melanocyte stimulating
30 hormone, neurotensin, adrenal peptide, parathyroid hormone and related peptides, somatostatin and related peptides, brain natriuretic peptide, calcitonin, corticotropin

releasing factor (CRF), cf. SEQ ID NO: 3 herein, thymosin, and urotensin; and homologous or otherwise related peptides and fragments of these and other polypeptides (e.g. EEID-CART₅₅₋₁₀₂, cf. SEQ ID NO: 2 herein), as long as the criteria of having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40 or from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, is fulfilled.

SUMMARY OF THE INVENTION

10

The above identified purpose is achieved with the method according to the present invention which comprises culturing a yeast which has reduced activity of Yap3 protease (Yap3p) or a homologue thereof and has been transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40, or from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, such as glucagon or glucagon like peptides; and isolating said polypeptides. Preferably, the yeast cells lack Yap3p activity through disruption of the YAP3 gene.

20

Using a YAP3 disrupted yeast strain for the production of polypeptides having from 1-70 amino acids, preferably from 1-40, and more preferably from 10-30 amino acids in the polypeptide chain, and having no more than one disulphide bonds in the structure such as polypeptides encoded by the glucagon precursor gene including glucagon, GRPP, GLP-1, GLP-2, and their functional analogues thereof result in a remarkably improved yield of up to about 2-fold and even 10-fold compared to the yield from the corresponding YAP3 wild-type yeast strain. It has been found that using a YAP3 disrupted yeast strain for the production of heterologous polypeptides having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40 or from 25-35 amino acids, in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, such as polypeptides

30

encoded by the glucagon precursor gene including glucagon, GRPP, GLP-1, GLP-2, and their functional analogues thereof or CRF, e.g. as shown in SEQ ID NO:3 herein, or truncated and/or N-terminally extended forms of CART, preferably EEID-CART₅₅₋₁₀₂ as shown in SEQ ID NO:2 herein, result in a remarkably improved yield of the
5 heterologous polypeptide of up to about 2-fold and even 10-fold compared to the yield obtained from the corresponding YAP3 wild-type yeast strain. Another advantage of using the method of the invention for production of heterologous polypeptides is that the secreted product has an improved homogeneity due to a reduced degree of proteolytic degradation.

10

The present inventors have also found that the use of a diploid YAP3 disrupted yeast in the method of the invention results in a significantly higher production level of secreted heterologous polypeptide which is about 2-fold and even 9-fold higher compared to the yield level from the corresponding wild-type haploid yeast.

15

Suitably, the yeast is *S. cerevisiae* which lacks a functional YAP3 gene. However, other yeast genera may have equivalent proteases, i.e. homologues of Yap3p, e. g. the genera *Pichia* and *Kluyveromyces* as shown in WO 95/23857 and Clerc et al. (J. Chromat. B. 662: 245-259, 1994). A gene is regarded as a homologue, in general, if
20 the sequence of the translation product has greater than 50% sequence identity to Yap3p. Komano and Fuller (Proc. Natl. Acad. Sci. USA 92: 10752-10756, 1995) has identified the Mkc7 aspartyl protease from *S. cerevisiae* which is closely related to Yap3p (53% identity). Other aspartyl proteases of *Saccharomyces* include the gene products of PEP4, BAR1, and of open reading frames, the sequences of which are
25 partially homologous with the YAP3 open reading frame, such as YAP3-link (coded by GenBank acc. No. X89514: pos. 25352-26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116). According to recently accepted yeast genome nomenclature the yeast gene names YAP3, YAP3 link, YIV 9, NO 4, and MKC 7 used herein correspond to the yeast open
30 reading frame YLR120C, YLR121C, YIR039C, YDR349C, and YDR144C.

respectively. Furthermore, the gene product of open reading frame YGL259W is included among the yeast aspartyl proteases.

Examples of yeasts include *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*,
5 *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida sp.*, *Candida utilis*, *Candida cacaui*, *Geotrichum sp.*, and *Geotrichum fermentans*.

A suitable means of eliminating the activity of a protease is to disrupt the host gene
10 encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease including regulatory and/or coding regions, or, alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations. Other methods which may be suitable for down regulation of the protease include the introduction of antisense and/or
15 ribozyme constructs in the yeast, e.g. Atkins et al. (Antisense and Development 5: 295-305, 1995) and Nasr et al. (Mol. Gen Genet 249: 51-57, 1995). One preferred method of disrupting the YAP3 gene in the yeast strain used in the method of the present invention are described by Rothstein (Method in Enzymol, 194: 281-301, 1991).

20

The expression "glucagon or glucagon like peptides" as used herein may be of human origin or from other animals and recombinant or semisynthetic sources and include all members of the glucagon family, such as GRPP (glicentine related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2),
25 including truncated and/or N-terminally extended forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y, GLP-2 A19T+34Y, GLP-2 A2G and GLP-2 A19T, and other analogues having from 1 to 3 amino acid changes, additions and/or deletions. The cDNA used for expression of the polypeptide according to the invention include codon optimised forms for expression in yeast.

30

Throughout the description and claims is used one and three letter codes for amino acids in accordance with the rules approved (1974) by the IUPAC-IUB Commission on Biochemical Nomenclature, vide Collected Tentative Rules & Recommendations of the Commission on Biochemical Nomenclature IUPAC-IUB, 2nd ed., Maryland, 1975.

5

A further aspect of the invention is a culture of yeast cells transformed with a hybrid vector containing a polynucleotide sequence, preferably a DNA sequence, encoding a polypeptide having up to 55 amino acids, preferably from 10 to 50 amino acids, more preferably from 15 to 40, or preferably from 20 to 30 amino acids, most preferably from
10 25 to 35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, said polynucleotide sequence or DNA sequence being operably linked to a polynucleotide sequence or DNA sequence encoding a yeast promoter and a leader sequence (pro sequence or prepro sequence) and/or other polynucleotide sequences or DNA
15 sequences that are necessary for said polypeptide to be expressed in and secreted from the yeast, said culture of yeast cells being characterized in that the cells have reduced Yap3p activity, preferably through a disruption of the YAP3 gene, and said culture of yeast cells being a culture of haploid or polyploid, preferably diploid, yeast cells.

20

In another aspect the invention provides a culture of yeast cells containing a polynucleotide sequence encoding a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40 or preferably from 20-30 amino acids, most preferably from 25 to 35 amino acids, and having from 0 to 3
25 disulphide bonds, preferably one or less disulphide bonds in the structure, and a second polynucleotide sequence encoding a secretion signal causing said polypeptide to be expressed in and secreted from the yeast, characterized in that the yeast cells have reduced Yap3 protease activity. Preferably, the yeast cells are diploid yeast cells transformed with a hybrid vector comprising said polynucleotide sequences, and
30 preferably the yeast cells lack Yap3p activity which may conveniently be obtained through disruption of the YAP3 gene.

- The DNA encoding the polypeptides having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bonds in the structure, may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration on host chromosome(s) is desired.
- Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.
- If integration is desired, the DNA is inserted into an yeast integration plasmid vector, such as pJJ215, pJJ250, pJJ236, pJJ248, pJJ242 (Jones & Prakash, Yeast 6: 363, 1990) or pDP6 (Fleig et al. Gene 46:237, 1986), in proper orientation and correct reading frame for expression, which is flanked with homologous sequences of any non-essential yeast genes, transposon sequence or ribosomal genes. Preferably the flanking sequences are yeast protease genes or genes used as a selective marker. The DNA is then integrated on host chromosome(s) by homologous recombination occurred in the flanking sequences, by using standard techniques shown in Rothstein (Method in Enzymol, 194: 281-301, 1991) and Cregg et al. (Bio/Technol. 11:905-910, 1993).
- Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the polypeptides to be produced according to the method of the invention, preferred examples of polypeptides being glucagon, glucagon like peptides, CRF and

EEID-CART₅₅₋₁₀₂, or their functional analogues, which can then be recovered, as is known.

Useful yeast plasmid vectors include the POT (Kjeldsen et al. Gene 170: 107-112, 1996) and YEp13, YEp24 (Rose and Broach, Methods in Enzymol. 185: 234-279, 1990), and pG plasmids (Schena et al. Methods in Enzymol. 194: 289-398, 1991).

Methods for the transformation of *S. cerevisiae* include the spheroplast transformation, lithium acetate transformation, and electroporation, cf. Methods in Enzymol. Vol. 194 (1991). Preferably the transformation is as described in the examples herein.

Suitable promoters for *S. cerevisiae* include the MF α 1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CYC1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoter. A suitable promoter in the genus *Pichia* is the AOX1 (methanol utilisation) promoter.

The transcription terminal signal is preferably the 3' flanking sequence of a eucaryotic gene which contains proper signal for transcription termination and polyadenylation. Suitable 3' flanking sequences may, e.g. be those of the gene naturally linked to the expression control sequence used, i.e. corresponding to the promoter.

The DNA constructs that are used for providing secretory expression of the polypeptide according to the invention comprise a DNA sequence that includes a leader sequence linked to the polypeptide by a yeast processing signal. The leader sequence contains a signal peptide ("pre-sequence") for protein translocation across the endoplasmic reticulum and optionally contains an additional sequence ("pro-sequence"), which may or may not be cleaved within yeast cells before the polypeptide is released into the surrounding medium. Useful leaders are the signal peptide of mouse α -amylase, *S. cerevisiae* MF α 1, YAP3, BAR1, HSP150 and *S. kluyveri* MF α signal peptides and prepro-sequences of *S. cerevisiae* MF α 1, YAP3, PRC, HSP150,

and S. kluyveri MF α and synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666. Furthermore, the polypeptides to be produced according to the method of the invention may be provided with an N-terminal extension as described in WO 95/35384.

5

The invention also relates to a method of preparing a yeast having reduced Yap3p activity comprising the steps of a) providing a hybrid plasmid containing a part of the YAP3 gene and suitable for transformation into a yeast cell, b) disrupting the YAP3 gene by deleting the fragment of YAP3 and inserting the URA3 gene instead to obtain
10 a $\Delta yap3::URA3$ gene disruption plasmid, c) providing a yeast $\Delta ura3$ deletion mutant, d) transforming said mutant with said plasmid, and e) selecting the $\Delta yap3::URA3$ deletion mutants on a medium without uracil. Further the invention relates to a method of preparing a yeast having reduced Yap3p activity using antisense technology.

15 Moreover, the polypeptides to be produced according to the method of the invention may conveniently be expressed coupled to an N- or C-terminal tag or as a precursor or fusion protein although the total length of the expressed polypeptide may exceed a total of 55 or 70 amino acids.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the construction of the pS194 plasmid.

Fig. 2 shows the construction of plasmids pME834 and pME1389.

Fig. 3 is a restriction map of the human glucagon expression plasmid pMT703.

25 Fig. 4 is a restriction map of the human GLP-1(7-37) expression plasmid pLaC253.

Fig. 5 is a restriction map of the human GLP-2 expression plasmid pKV210.

Fig. 6 is a restriction map of the pME973 plasmid, containing the genes encoding the HO (homothallism) endonuclease and Ura3p inserted into the YEp13 plasmid.

30

DETAILED DESCRIPTION OF THIS INVENTION

Preferred embodiments of this invention are described in Table 1 below. Having knowledge of the art, it will be obvious to a skilled person to produce other polypeptides having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure and their functional analogues by the method of the present invention using similar constructs.

10

Table 1

Presequence (signal)	Prosequence	Heterologous protein
MF α 1 (1-19)	MF α 1(20-85)	Glucagon
MF α 1 (1-19)	MF α 1(20-81)MAKR	DDDDK-Glucagon
MF α 1 (1-19)	MF α 1(20-85)	GLP-1(7-37)
YAP3(1-21)	LA19 ¹ KR	GLP-1(7-35)R36A
spx3 ²	LaC212	GRPP
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2
HSP150(1-18)	HSP150(19-67)- WIIAENTTLANVAMAKR	GLP-2
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue F22Y
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue A19T, +34Y
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue A19T
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue A2G
MF α 1 (1-19)	MF α 1(20-81)MAXKR X= peptide bond or Y or S or K or E or ARS	Glucagon or Calcitonin

¹ LA19, cf. SEQ ID NO:1 herein and WO 95/34666, ² spx3-LaC212, cf. WO 89/02463 and WO 90/10075.

15

The Genetic background of *S. cerevisiae* strains used herein is as follows:

	E11-3C	MAT α YAP3 pep4-3 Δ tpi::LEU2 leu2 URA3
5	SY107	MAT α YAP3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
	ME1487	MAT α Δ yap3::URA3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
	ME1656	MAT α Δ yap3::ura3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
10	ME1684	MAT α Δ yap3::URA3:: Δ ylr121c pep4-3 Δ tpi::LEU2 leu2 Δ ura3
	ME1695	MAT α Δ yap3::ura3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
15	ME1719	MAT α / α Δ yap3::URA3/ Δ yap3::ura3 pep4-3/pep4-3 Δ tpi::LEU2/ Δ tpi::LEU2 leu2/leu2 Δ ura3/ Δ ura3
	MT663	MAT α / α YAP3/YAP3 pep4-3/pep4-3 Δ tpi::LEU2/ Δ tpi::LEU2 leu2/leu2 URA3/URA3 HIS4/his4
20		

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the fore-going description and in the following examples may, both separately and in
 25 any combination thereof, be material for realizing the invention in diverse forms thereof.

Example 1

30 Δ yap3::URA3 gene disruption

The Δ ura3 deletion mutation was constructed as follows:

pJJ244 (pUC18 containing a 1.2 kb HindIII fragment of the URA3 gene) was digested with Styl and filled in with Klenow polymerase and self ligated. The resulting
 35 plasmid designated pS194 contains a 84bp of Styl-Styl fragment deletion of the URA3 gene, cf. Fig.1.

The $\Delta yap3::URA3$ gene disruption plasmid pME1389 was constructed as follows:

The 2.6kb *SacI*-*PstI* fragment which contains the YAP3 gene in pME768 (Egel-Mitani et al. Yeast 6: 127-137, 1990) was inserted in 2.6 kb of the *SacI*-*PstI* fragment of pIC19R (Marsh et al. Gene 32: 481-485, 1984). The resulting plasmid is pME834.

- 5 pME834 was digested with *HindIII* to form a deletion of the 0.7 kb YAP3 fragment and the 1.2 kb *HindIII* fragment of the URA3 gene (Rose et al. Gene 29: 113-124, 1984) was inserted instead. The resulting plasmid is pME1389. The construction of plasmids pME834 and pME1389 is shown in Fig. 2 in diagrammatic form.

- 10 *S. cerevisiae* strain E11-3C ($MAT\alpha$ YAP3 pep4-3 $\Delta tpi::LEU2$ leu2 URA3), cf. ATCC 20727, US pat. 4766073, was transformed with linearized pS194 (*BsgI* digested) to make $\Delta ura3$ deletion mutation. By selection on 5-FOA (5-fluoro-orotic acid) containing minimal plates, the $\Delta ura3$ mutant designated SY107 was obtained.

- 15 The strain SY107 ($MAT\alpha$ YAP3 pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$), was then transformed with pME1389 previously being cut by *Sall* and *SacI*, and 3kb fragment of $\Delta yap3::URA3$ was isolated for the transformation. $\Delta yap3::URA3$ deletion mutants were selected on minimal plates without uracil. URA3 transformants were characterized by PCR and Southern hybridisation to confirm the correct integration of
- 20 the $\Delta yap3::URA3$ fragment in the YAP3 locus. ME1487 was isolated as a $\Delta yap3::URA3$ deletion mutant ($MAT\alpha$ $\Delta yap3::URA3$ pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$).

Example 2

25

Construction of a diploid $\Delta yap3/\Delta yap3$ strain

- ME1487 was mutagenized by using EMS (methane-sulfonic acid ethylester) and *ura3* mutants were selected on plates containing 5-FOA. One of the selected
- 30 isolates, ME1656 was then subjected to mating type switch (Herskowitz and Jensen, Methods in Enzymol. 194: 132-146, 1991) by transient transformation with pME973

shown in Fig. 6. pME973 contains the genes encoding the HO (homothallism) endonuclease and URA3 inserted into the YEp13 plasmid (Rose and Broach, Methods in Enzymol. 185: 234-279, 1990). From transient transformants, ME1695 was selected as the haploid strain, which had switched from MAT α to MATa, and
5 have the following genetic background: MATa Δ yap3::ura3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3.

ME1695 was then crossed with ME1487 by micromanipulation (Sherman and Hicks, Methods in Enzymol. 194: 21-37, 1991) in order to get Δ yap3/ Δ yap3 diploids. From
10 the resulting diploids, ME1719 was selected as the strain with the following genetic background:

MATa/ α Δ yap3::ura3/ Δ yap3::URA3 pep4-3/pep4-3 Δ tpi::LEU2/ Δ tpi::LEU2 leu2/leu2 Δ ura3/ Δ ura.

15

Example 3

Construction of a Δ yap3::URA3:: Δ ylr121c double disruption strain

20 In order to make a one-step gene disruption strain of the two closely linked genes encoding YAP3 and YLR121C, the following two oligonucleotide primers were synthesized:

P1 Length 57bp: YLR121C/URA3 primer

25

5'-GAT CGA ACG GCC ATG AAA AAT TTG TAC TAG CTA ACG
AGC AAA GCT TTT CAA TTC AAT-3'

P2 Length 57bp: YAP3/URA3 primer

30

5'-CCA GAA TTT TTC AAT ACA ATG GGG AAG TTG TCG TAT
TTA TAA GCT TTT TCT TTC CAA-3'

P1 and P2 each contains 40 nucleotides corresponding to sequences within the
coding region of YLR121C and YAP3, respectively, as well as a HindIII site
(AAGCTT) and 12 nucleotides corresponding to sequences flanking the URA3 gene
(YEL021W). P1 and P2 were used for PCR using the URA3 gene as template. The
resulting 1248bp PCR fragment contains the URA3 selective marker flanked with 40
nucleotides derived from the YAP3 or YLR121C encoding regions. The PCR
fragment was then transformed into ME1655, and $\Delta yap3::URA3::\Delta ylr121c$ deletion
mutants were selected and characterized as described in Example 1. ME1684 was
isolated as a $\Delta yap3::URA3::\Delta ylr121c$ mutant with the following genetic background:
MAT α $\Delta yap3::URA3::\Delta ylr121c$ pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$.

Example 4

Transformation into yeast

In order to make yeast competent cells, yeast haploid strains SY107 and ME1487 or
the diploid ME1719 strain were cultivated in 100ml YPGGE medium (1% yeast
extract, 2% peptone, 2% glycerol, 2% galactose, 1% ethanol) to OD₆₀₀ = 0.2. Cells
were harvested by centrifugation at 2000rpm for 5 min. and washed once by 10ml
H₂O. Cells were resuspended in 10ml SED (1M sorbitol, 25mM Na₂EDTA pH8,
6.7mg/ml dithiothreitol) and incubated at 30°C for 15min. Cells were harvested by
centrifugation and resuspended in 10ml SCE (1M sorbitol, 0.1M Na-citrate, 10mM
Na₂EDTA, pH5.8) + 2mg Novozyme SP234 and incubated at 30°C for 30 min. After
cells were harvested by centrifugation and washed once by 10ml 1.2M sorbitol and
subsequently by 10ml CAS (1M sorbitol 10mM CaCl₂, 10mM Tris-HCl pH7.5), cells
were harvested by centrifugation and resuspended finally in 2ml CAS. Competent
cells were frozen in portion of 100 μ l per Eppendorf tube at -80°C.

Transformation was made as follows: Frozen competent cells (100 μ l) were warmed up quickly and 1 μ g plasmid DNA were added. Cells were incubated at room temp. for 15 min. and 1ml PEG solution (20% polyethyleneglycol 4000, 10mM CaCl₂, 10mM Tris-HCl pH7.5) was added. After 30min. at room temperature, cells were

5 harvested by centrifugation at 2000rpm for 15min. and resuspended in 100 μ l SOS (1M sorbitol, 1/2 vol. YPGGE, 0.01% uracil, 7mM CaCl₂). After incubating at 30°C for 2 hours, cells were centrifuged and resuspended in 0.5ml 1M sorbitol. Cells were then spread on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) together with 6ml of top agar (YPD containing 2.5% agar). Plates were incubated at

10 30°C for 3 to 5 days until transformants appear.

Example 5

Heterologous protein expression plasmid

15

Yeast-E.coli shuttle vector used in the following examples contains a heterologous protein expression cassette, which includes a DNA sequence encoding a leader sequence followed by the heterologous polypeptide in question operably placed in between the TPI promoter and TPI terminator of *S. cerevisiae* in a POT plasmid

20 (Kjeldsen et al. 1996, *op. cit.*). The leader sequences are the MF α 1 prepro-sequence and modification thereof. Examples are shown as follows:

Table 2

Presequence (signal)	Prosequence	Heterologous protein	Plasmid
MF α 1(1-19)	MF α 1(20-85)	Glucagon	pMT703 Fig. 3
MF α 1(1-19)	MF α 1(20-85)	GLP-1(7-37)	pLaC253 Fig. 4
MF α 1(1-19)	MF α 1(20-81)MAKR	GLP-2	pKV210 Fig. 5
MF α 1(1-19)	MF α 1(20-81)MAKR	CRF ₁₋₄₁	pKV241
MF α 1(1-19)	MF α 1(20-81)MAKR	EEID-CART ₅₅₋₁₀₂	pSX647

Example 6**5 Expression of glucagon**

Human glucagon expression plasmid pMT703, cf. Figure 3, was transformed into three strains, such as, YAP3 disrupted haploid strain ME1487($\Delta yap3$), YAP3 disrupted diploid strain ME1719 ($\Delta yap3/\Delta yap3$) and YAP3 wild-type strain SY107 (YAP3 WT). Transformants were selected by glucose utilization as a carbon source in YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% glucose, 2% agar). ME1532 and YES1746 are pMT703 transformants obtained from ME1487 ($\Delta yap3$) and ME1719 ($\Delta yap3/\Delta yap3$), respectively, whereas ME1530 is the pMT703 transformant obtained from SY107 (YAP3 WT). Transformants were cultivated in 5ml YPD liquid medium at 30°C for 3 days with shaking at 200rpm. Culture supernatants were obtained after centrifugation at 2500rpm for 5 min. and 1ml supernatants were analyzed by reverse phase HPLC. Production levels, shown in Table 3, were average value of cultures from 2 independently isolated transformants (Exp.1) or values from a mixculture of 3 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses showed that ME1532 or YES1746 produced approx. 4 to 6 times more glucagon than ME1530.

HPLC setting for glucagon detection

HPLC-Column: 4 x 250 mm Novo Nordisk YMC-OdDMeSi C18 5 μ m
Column temp.: 50°C
Flowrate: 1 ml/min
HPLC solvents:
A: 10 % (v/v) acetonitrile in 0.2 M Na_2SO_4 , 0.04 M H_3PO_4 pH adjusted to 2.3 with ethanolamine

B: 50 % (v/v) acetonitrile in water

Inj. vol: 150 μ l

5

Glucagon was eluted from the HPLC columns with 23.6 % acetonitrile to 32.9 % acetonitrile in 40 min.

10

Table 3

TRANSFORMANT	HOST	PLASMID	GLUCAGON LEVEL	
			Exp.1	Exp.2
ME1530	SY107	pMT703	100%	100%
ME1532	ME1487	pMT703	596%	469%
YES1746	ME1719	pMT703	ND	587%

15 **Example 7**

Expression of GLP-1(7-37)

Human GLP-1(7-37) expression plasmid pLaC253, cf. Figure 4, was transformed into
20 ME1487(Δ yap3), ME1719 (Δ yap3/ Δ yap3) and SY107 (YAP3 WT). Transformants
were selected and analysed as described in Example 6. ME1535 and YES1823 are
the pLaC253 transformants obtained from ME1487(Δ yap3) and ME1719
(Δ yap3/ Δ yap3), respectively, whereas ME1534 is the pLaC253 transformant
obtained from SY107 (YAP3 WT). Production levels, shown in Table 4, were average
25 value of cultures from 2 independently isolated transformants (Exp.1) or values from
a mixculture of 3 transformants (Exp.2), and were normalised so that the haploid
YAP3 wild-type level was taken as 100%. HPLC analyses showed that ME1535 or
YES1823 produced approx. 2 to 3 times more GLP-1(7-37) than ME1530.

HPLC settings for GLP-1(7-37) detection:

As described in Example 6, except that GLP-1 was eluted from HPLC columns with 31.2% acetonitrile to 41.2% acetonitrile in 40 min.

5

Table 4

TRANSFORMANT	HOST	PLASMID	GLP-1(7-37) LEVEL	
			Exp. 1	Exp. 2
ME1534	SY107	pLaC253	100%	100%
ME1535	ME1487	pLaC253	287%	
YES1823	ME1719	pLaC253	ND	161%

10

Example 8**Expression of GLP-2**

15 Human GLP-2 expression plasmid pKV210, cf. Figure 5, was transformed into ME1487 ($\Delta yap3$), ME1719 ($\Delta yap3/\Delta yap3$) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1615 and YES1827 are the pKV210 transformants obtained from ME1487 ($\Delta yap3$) and ME1719 ($\Delta yap3/\Delta yap3$), respectively, whereas ME1614 is the pKV210 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 5 were average value of
20 cultures from 2 independently isolated transformants (Exp.1) or values from a mixture of 3 transformants (Exp.2), and were normalized so that the YAP3 wild-type level was taken as 100%. HPLC analyses showed that ME1615 and YES1827 produced approx. 6 to 11 times more GLP-2 than ME1614.

25

HPLC settings for GLP-2 detection:

HPLC-Column: Vydac 214TP54 Column

Flowrate: 1 ml/min

30

HPLC solvents:

A: 0.1% TFA
B: 0.07% TFA in acetonitrile

5

GLP-2 was eluted from the HPLC columns with 0.07% TFA in 20% to 80% acetonitrile in 60 min.

10

Table 5

TRANSFORMANT	HOST	PLASMID	GLP-2 LEVEL	
			Exp. 1	Exp. 2
ME1614	SY107	pLaC210	100%	100%
ME1615	ME1487	pLaC210	1130%	682%
YES1827	ME1719	pLaC210	ND	675%

Example 9

15 Expression of CRF₁₋₄₁ (SEQ ID NO:3 herein)

Human Corticotropin Releasing Factor (CRF₁₋₄₁) expression plasmid pKV241 (equivalent to pKV210 Fig. 5 in which MF α 1-pre-pro(1-81)MAKR-GLP2 is substituted by MF α 1-pre-pro(1-81)MAKR-CRF₁₋₄₁) was transformed into
20 ME1487(Δ yap3), ME1719 (Δ yap3/ Δ yap3) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1813 and YES1810 are the pKV241 transformants obtained from ME1487(Δ yap3) and ME1719 (Δ yap3/ Δ yap3), respectively, whereas ME1812 is the pKV241 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 6, were values from a
25 mixture of 3 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses were performed as in example 8 and showed that ME1813 or YES1810 produced approx. 8 to 9 times more CRF₁₋₄₁ than ME1812.

30 Table 6

TRANSFORMANT	HOST	PLASMID	CRF ₁₋₄ LEVEL	
			Exp. 1	Exp. 2
ME1812	SY107	pKV241	ND	100%
ME1813	ME1487	pKV241	ND	834%
YES1810	ME1719	pKV241	ND	911%

Example 10

Expression of EEID-CART₅₅₋₁₀₂ (SEQ ID NO:2 herein)

5 N-terminal extended (EEID) fragment of Human Cocaine and Amphetamine regulated transcript (EEID-CART₅₅₋₁₀₂) expression plasmid pSX637 (equivalent to pKV210 Fig. 5, in which MF α 1-pre-pro(1-81)MAKR-GLP2 is substituted by MF α 1-pre-pro(1-81)MAKR-EEID-CART₅₅₋₁₀₂), was transformed into ME1487(Δ yap3),
 10 ME1719 (Δ yap3/ Δ yap3) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1817 and YES1820 are the pSX637 transformants obtained from ME1487(Δ yap3) and ME1719 (Δ yap3/ Δ yap3), respectively, whereas ME1816 is the pSX637 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 7, were values from a mixculture of 3
 15 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses were performed as in example 8 and showed that ME1817 or YES1820 produced approx. 2 to 3 times more EEID-CART₅₅₋₁₀₂ than ME1816.

20 **Table 7**

TRANSFORMANT	HOST	PLASMID	EEID-CART ₅₅₋₁₀₂ LEVEL	
			Exp. 1	Exp. 2
ME1816	SY107	pSX637	ND	100%
ME1817	ME1487	pSX637	ND	216%
YES1820	ME1719	pSX637	ND	282%

Example 11

Table 8 shows data for expression levels of human glucagon, GLP-1₍₁₋₃₇₎ and GLP-2 in ME1487 ($\Delta yap3$) transformed by expression plasmids with different pre-pro-sequences (leaders). Expression plasmids are as described in Fig. 5 except for the details given in Table 8. Expression yields are normalised so that the yield obtained in MT663 (YAP3/YAP3) transformants is set to 100%.

10 **Table 8**

Presequence (signal)	Prosequence	Heterologous protein	Expression Plasmid	Yeast transformant	Yield % ME1487
MF α 1(1-19)	MF α (20-81)MA KR	Glucagon	pKV 216	ME1652	267
YAP3 (1-21)	LA19-KR	Glucagon	pKV 225	ME1780	333
MF α 1(1-19)	MF α (20-81)MARS KR	Glucagon	pKV 217	ME1691	400
MF α 1(1-19)	MF α (20-81)MARK KR	Glucagon	pKV 223	ME1692	185
MF α 1(1-19)	MF α (20-81)MARE KR	Glucagon	pKV 238	ME1727	167
MF α 1(1-19)	MF α (20-81)MAERLE KR	Glucagon	pKV 237	ME1726	189
MF α 1(1-19)	MF α (20-81)MAKELE KR	Glucagon	pKV 236	ME1725	234
MF α 1(1-19)	MF α (20-81)MA KR	GLP-1 ₍₁₋₃₇₎	pKV 230	ME1718	189
MF α 1(1-19)	MF α (20-81)MA KR	GLP-2 A19T	pKV 219	ME1677	411
MF α 1(1-19)	MF α (20-81)MA KR	GLP-2 A2G	pKV 220	ME1678	360
MF α 1(1-19)	MF α (20-81)MA KR	GLP-2 F22Y	pKV 249	ME1781	280

SEQUENCE LISTING

5

INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gln Pro Ile Asp Asp Thr Glu Ser Asn Thr Thr Ser Val Asn Leu
1 5 10 15
20 Met Ala Asp Asp Thr Glu Ser Arg Phe Ala Thr Asn Thr Thr Leu
20 25 30
Ala Leu Asp Val Val Asn Leu Ile Ser Met Ala
25 35 40

30

INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 52 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Glu Ile Asp Ile Pro Ile Tyr Glu Lys Lys Tyr Gly Gln Val
1 5 10 15
45 Pro Met Cys Asp Ala Gly Glu Gln Cys Ala Val Arg Lys Gly Ala
20 25 30

24

Arg Ile Gly Lys Leu Cys Asp Cys Pro Arg Gly Thr Ser Cys Asn
35 40 45

Ser Phe Leu Leu Lys Cys Leu
5 50

INFORMATION FOR SEQ ID NO:3

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20 Ser Glu Glu Pro Pro Ile Ser Leu Asp Leu Thr Phe His Leu Leu
1 5 10 15

Arg Glu Val Leu Glu Met Ala Arg Ala Glu Gln Leu Ala Gln Gln
20 25 30

25 Ala His Ser Asn Arg Lys Leu Met Glu Ile Ile
35 40

CLAIMS

1. A method for the production of a short chain polypeptide in yeast, comprising culturing a yeast having reduced activity of Yap3 protease or a homologue thereof,
5 said yeast being transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bonds in the structure, and isolating
10 said polypeptides:
2. A method for the production of an open structured short chain polypeptide in yeast, comprising culturing a yeast having reduced activity of Yap3 protease or of a homologue thereof, said yeast being transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a polypeptide having
15 from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bonds in the structure, and isolating said polypeptides.
3. A method according to claim 1 or 2, wherein the yeast lacks Yap3 protease activity.
4. A method according to any one of claims 1 to 3, wherein the yeast is a diploid yeast.
- 20 5. A method according to any one of claims 1 to 4, wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida* sp., *Candida utilis*, *Candida cacaoi*, *Geotrichum* sp., and *Geotrichum fermentans*.
- 25 6. A method according to claim 5, wherein the yeast is *S. cerevisiae*.
7. A method according to any one of the preceding claims wherein the yeast additionally has reduced protease activity selected from the group of proteases coded for by BAR1, STE13, PRA, PRB, KEX1, PRC, CPS, and the YAP3 homologues MKC7, YAP3-link (coded by GenBank acc. No. X89514: pos.25352-

26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116) genes.

8. A method according to any one of claims 1 to 6 wherein the yeast additionally has reduced protease activity selected from the group of proteases coded for by STE13, PRA, PRB, KEX1, and PRC genes.
9. A method according to any one of claims 1 to 6, wherein the yeast additionally has reduced protease activity of the aspartyl protease encoded by the yeast open reading frame YGL259W or selected from the group of serine proteases coded for by the KEX2 gene and the yeast open reading frame YCR045C and YOR003W,
10. A method according to any one of the preceding claims wherein the yeast additionally has the pep4-3 mutation.
11. A method according to any one of the preceding claims, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a pre-sequence (signal) or a prepro-sequence linked in the proper reading frame to a second DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bonds in the structure, and a DNA sequence containing yeast transcription termination signals.
12. A method according to any one of claims 1 to 9, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a pre-sequence (signal) or a prepro-sequence linked in the proper reading frame to a second DNA sequence coding for a polypeptide having from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bonds in the structure, and a DNA sequence containing yeast transcription termination signals.

13. A method according to claim 11 or 12, wherein the yeast promoter is selected from the group consisting of the MF α 1 promoter, CYC1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoters.
14. A method according to claim 11 or 12, wherein the hybrid vector comprises a first DNA sequence encoding a signal peptide of mouse α -amylase, *S. cerevisiae* MF α 1, BAR1, YAP3 and HSP150 and *S. kluyveri* MF α signal peptides or prepro-sequences of *S. cerevisiae* MF α 1, YAP3, PRC, HSP150 and *S. kluyveri* MF α and/or synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666.
15. A method according to any one of the preceding claims wherein the DNA sequence encoding a polypeptide is selected from the group consisting of DNA sequences encoding glucagon family, such as GRPP (glucocorticotrophic hormone related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2), including truncated forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y, GLP-2 A19T+34Y, GLP-2 A2G and GLP-2 A19T, and other analogues having from 1 to 3 amino acid changes, additions and/or deletions.
16. A method according to any one of claims 1 to 14, wherein the DNA sequence encoding a polypeptide is selected from the group of DNA sequences encoding CRF and a truncated and/or N-terminally extended form of CART, preferably EEID-CART₅₅₋₁₀₂.
17. A culture of yeast cells containing a DNA sequence encoding a polypeptide having up to 55 amino acids, preferably from 10 to 50 amino acids, more preferably from 15 to 40, or preferably from 25 to 35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, and a second DNA sequence encoding a leader sequence causing said polypeptide to be secreted from the yeast, characterized in that said culture of yeast cells has reduced Yap3p activity.

18. A culture of yeast cells according to claim 17 which is transformed with a hybrid vector comprising said DNA sequence and said second DNA sequence.
19. A culture of yeast cells containing a DNA sequence encoding a polypeptide having from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bond in the structure, and a second DNA sequence encoding a leader sequence causing said polypeptide to be secreted from the yeast, characterized in that the yeast cells have reduced Yap3p activity.
20. A culture according to claim 19 which is transformed with a hybrid vector comprising said DNA sequence and said second DNA sequence.
21. A culture according to any one of claims 17 to 20, wherein the yeast cells lacks Yap3p activity.
22. A culture according to any one of claims 17 to 21, wherein the yeast cells are diploid.
23. A culture according to any one of claims 17 to 22, wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida sp.*, *Candida utilis*, *Candida cacaoi*, *Geotrichum sp.*, and *Geotrichum fermentans*.
24. A culture according to the preceding claim, wherein the yeast is *S. cerevisiae*.
25. A culture according to any one of the claims 17 to 24, wherein the yeast cells additionally have reduced protease activity selected from the group of proteases coded for by the BAR1, STE13, PRA, PRB, KEX1, PRC, CPS, and the YAP3 homologues MKC7, YAP3-link (coded by GenBank acc. No.X89514: pos. 25352-26878), YIV9 (Swiss Prot acc. No. P40583), aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116), genes.
26. A culture according to any one of the claims 17 to 24, wherein the yeast cells additionally have reduced protease activity selected from the group of proteases coded for by the STE13, PRA, PRB, KEX1, and PRC genes.

27. A culture according to any one of the claims 17 to 24, wherein the yeast additionally has reduced protease activity of the *aparatyl* protease encoded by the yeast open reading frame YGL259W or selected from the group of serine proteases coded for by the KEX2 gene and the yeast open reading frame YCR045C and YOR003W.
- 5 28. A culture according to any one of claims 17 to 27, wherein the yeast cells additionally have the *pep4-3* mutation.
29. A culture according to any one of claims 17 to 28, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a presequence (signal) or a preprosequence linked in
10 the proper reading frame to a second DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, and a DNA sequence containing yeast transcription termination
15 signals.
30. A culture according to any one of claims 17 to 28, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a presequence (signal) or a preprosequence linked in the proper reading frame to a second DNA sequence coding for a polypeptide
20 having from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bond in the structure, and a DNA sequence containing yeast transcription termination signals.
31. A culture according to claim 29 or 30, wherein the yeast promoter is selected from
25 the group consisting of the $MF\alpha 1$ promoter, CYC1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoters.
32. A culture according to claim 29 or 30, wherein said first DNA sequence is a signal
30 peptide of mouse α -amylase, *S. cerevisiae* $MF\alpha 1$, BAR1, YAP3 and HSP150 and *S.*

kluyveri MF α signal peptides or prepro-sequences of *S. cerevisiae* MF α 1, YAP3, PRC, HSP150 and *S. kluyveri* MF α and/or synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666.

33. A culture according to any one of claims 17 to 32, wherein the DNA sequence
5 encoding a polypeptide is a DNA sequence encoding a polypeptide of the glucagon family, such as GRPP (glicentine related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2), including truncated forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y, GLP-2 A19T+34Y, GLP-2 A2G and GLP-2 A19T, and other analogues having from
10 1 to 3 amino acid changes, additions and/or deletions.
34. A culture according to any one of claims 17 to 32, wherein the DNA sequence encoding a polypeptide is selected from the group of DNA sequences encoding CRF and a truncated and/or N-terminally extended CART, preferably EEID-CART₅₅.
- 102
- 15 35. A method of preparing a yeast having reduced Yap3p activity comprising the steps of a) providing a hybrid plasmid containing the a part of the YAP3 gene and suitable for transformation into a yeast cell, b) disrupting the YAP3 gene by deleting the fragment of YAP3 and inserting a URA3 gene instead to obtain a $\Delta yap3::URA3$ gene disruption plasmid, c) providing a yeast $\Delta ura3$ deletion mutant, d) transforming
20 said mutant with said plasmid, and e) selecting $\Delta yap3::URA3$ deletion mutants on a medium without uracil.
36. A method of preparing a yeast having reduced Yap3p activity using antisense technology.
37. A method according to claims 35 or 36 wherein the yeast is *S. cerevisiae*.
- 25 38. Any novel feature or combination of features described herein.

1/6

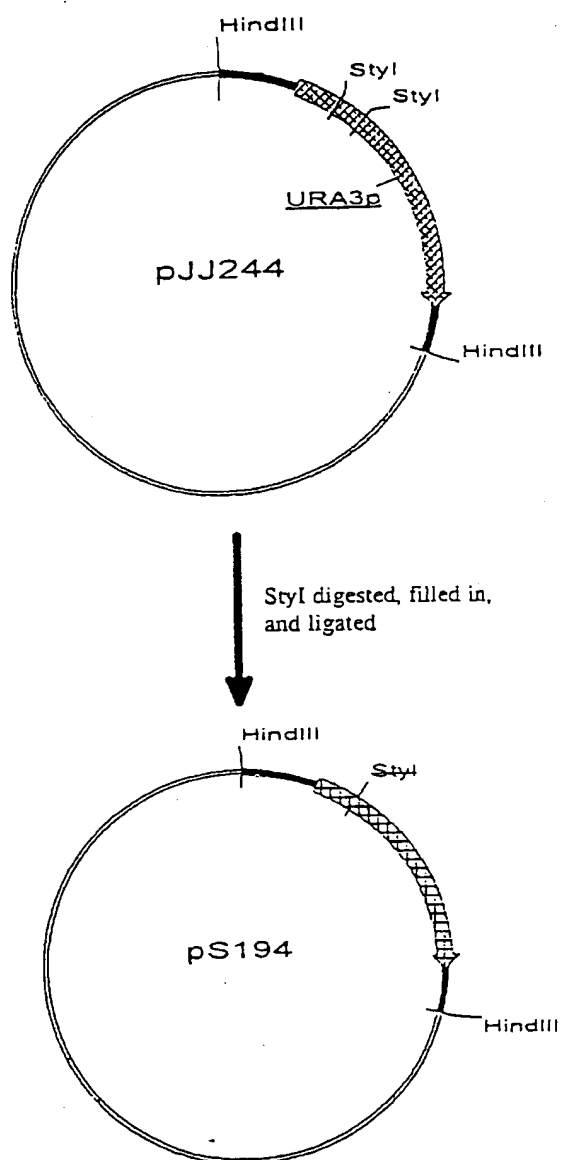


Fig. 1

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2/6

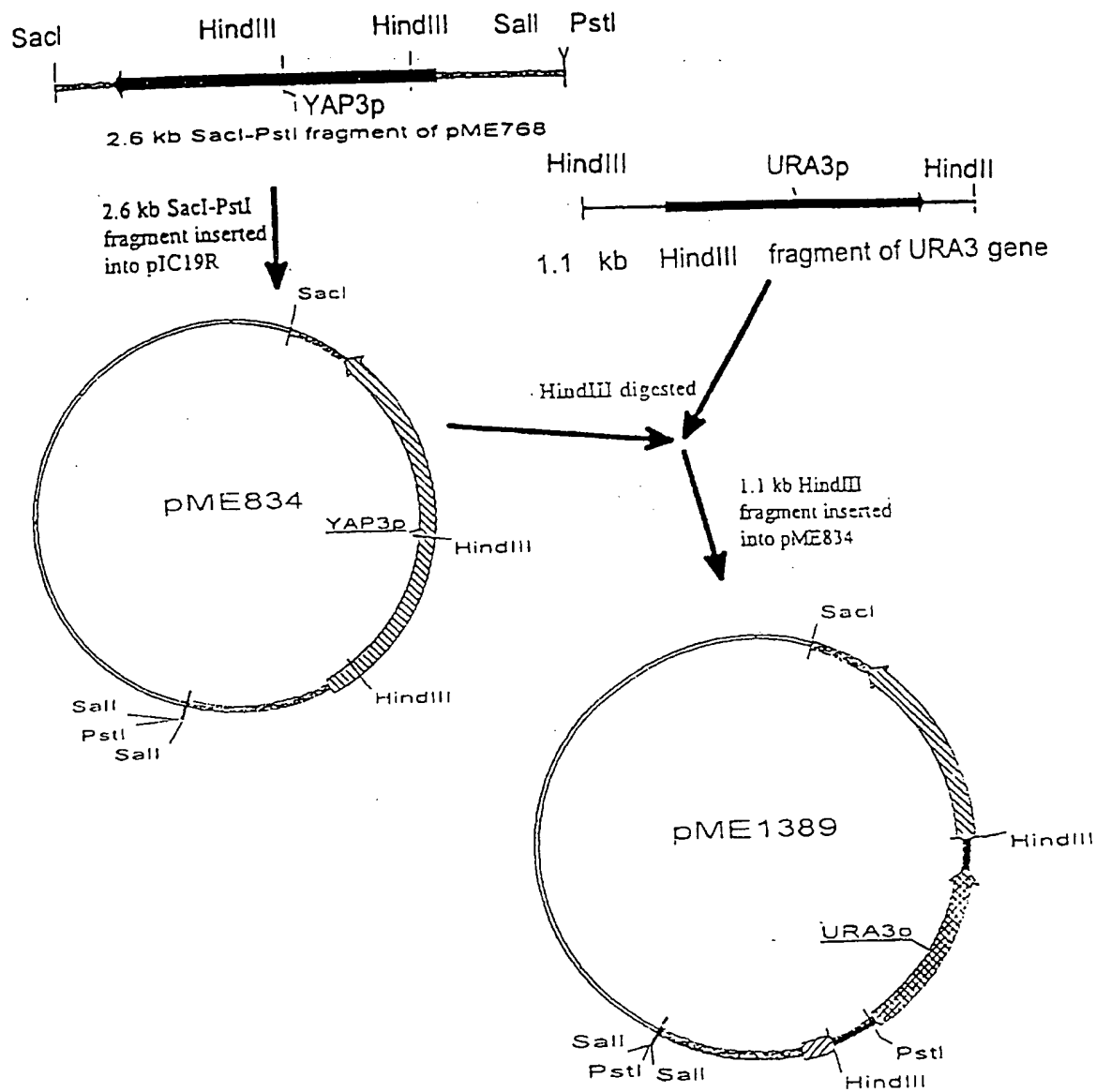


Fig. 2

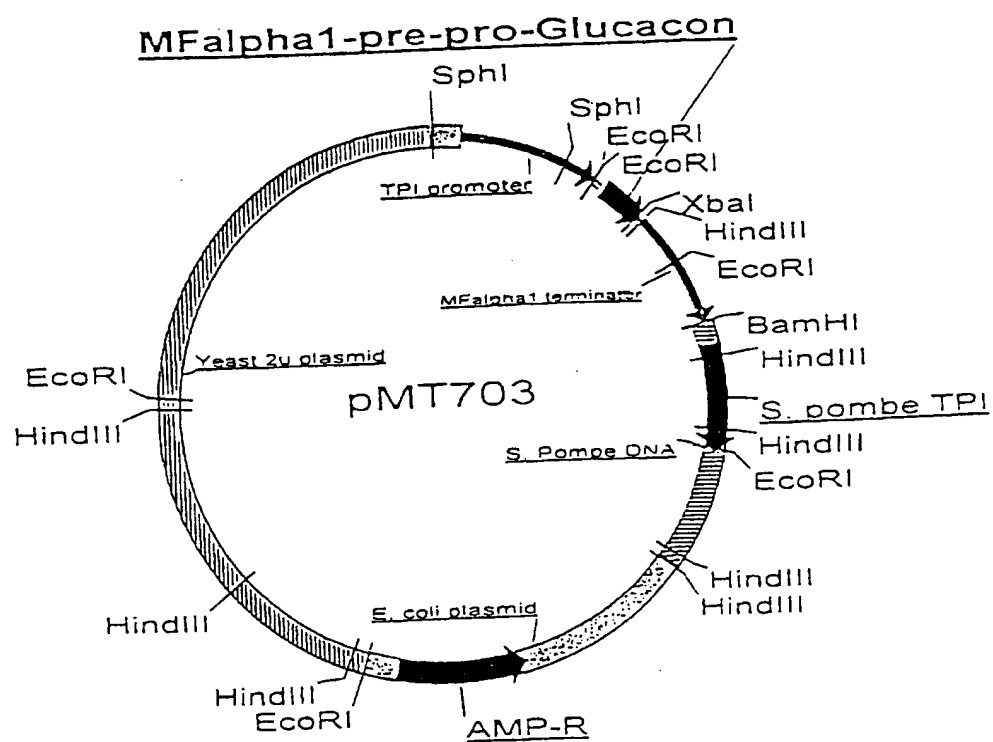


Fig. 3

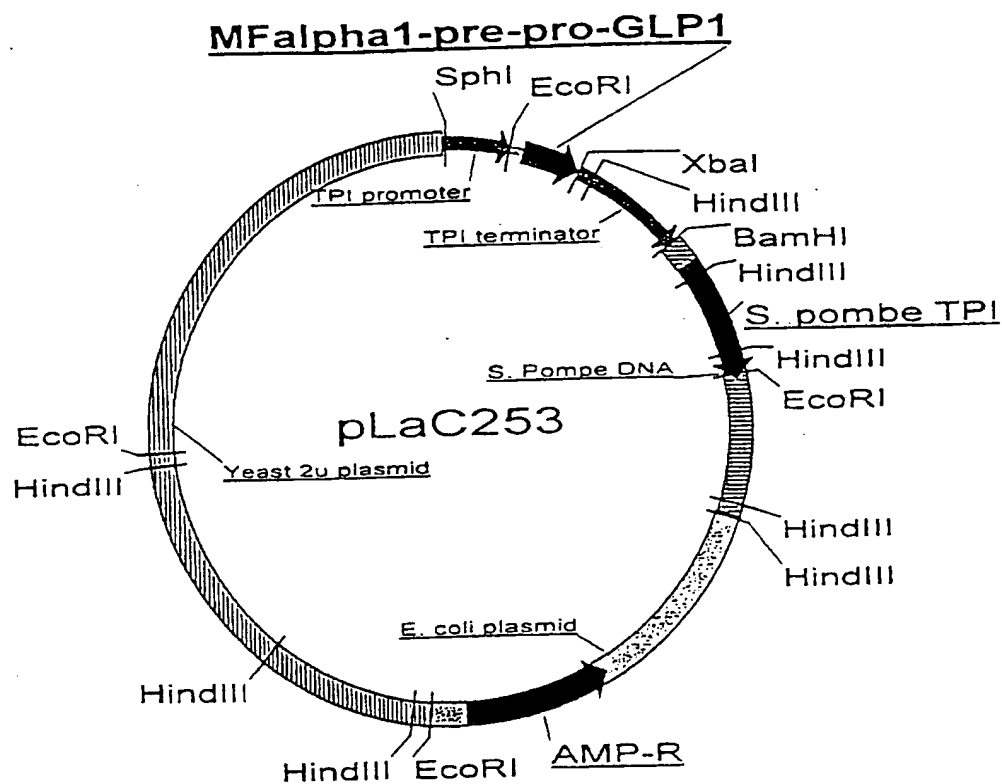


Fig. 4

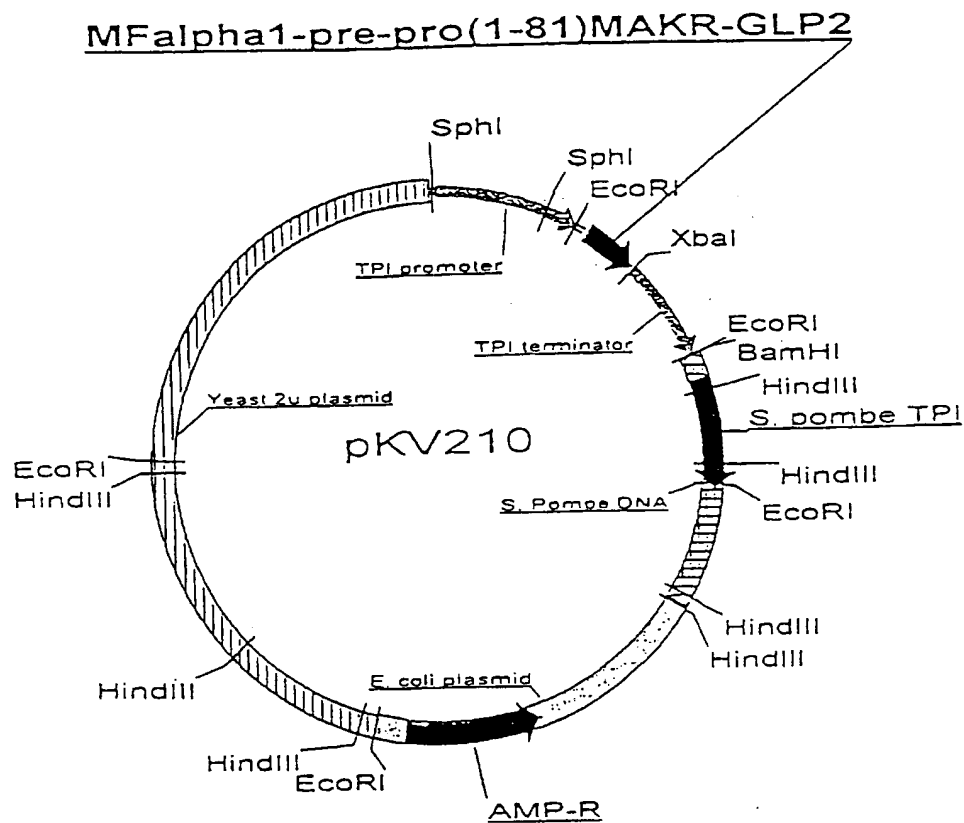


Fig. 5

6/6

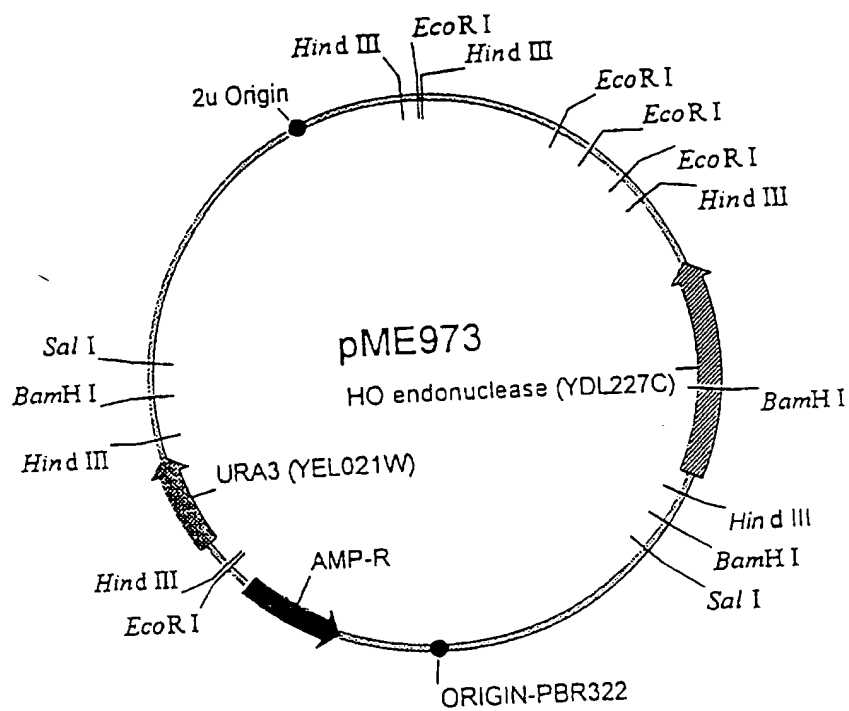


Fig. 6

INTERNATIONAL SEARCH REPORT

Int. l. application No.

PCT/DK 97/00298

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: C12N 1/19, C07K 14/605 // C12N 15/81 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C12N, C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, BIOSIS DBA, SCISEARCH, MEDLINE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9523857 A1 (DELTA BIOTECHNOLOGY LIMITED), 8 Sept 1995 (08.09.95)	1-38
	--	
A	Dialog Information Service, file 154, Medline, Dialog accession no. 06480997, Medline accession no. 90382674, Gabrielsen OS et al: "Efficient secretion of human parathyroid hormone by Saccharomyces cerevisiae", Gene (NETHERLANDS) Jun 15 1990, 90 (2) p255-62	1-38
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<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00298

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Service, file 154, Medline, Dialog accession no. 06460032, Medline accession no. 90224362, Egel-Mitani M. et al: "A novel aspartyl protease allowing KEX2-independent MF alpha propheremone processing in yeast", Yeast (ENGLAND) Mar-Apr 1990, 6 (2) p127-37</p> <p>-- -----</p>	1-38

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/DK 97/00298

Patent document cited in search report			Publication date		Patent family member(s)		Publication date	
WO	9523857	A1	08/09/95		AU	1818395 A	18/09/95	
					CA	2183241 A	08/09/95	
					EP	0749478 A	27/12/96	
					GB	2301365 A,B	04/12/96	
					GB	9404270 D	00/00/00	
					GB	9616724 D	00/00/00	

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 1/19, C07K 14/605 // C12N 15/81	A1	(11) International Publication Number: WO 98/01535 (43) International Publication Date: 15 January 1998 (15.01.98)
(21) International Application Number: PCT/DK97/00298 (22) International Filing Date: 4 July 1997 (04.07.97) (30) Priority Data: 0749/96 5 July 1996 (05.07.96) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): EGEL-MITANI, Michi [DE/DK]; Gøngesletten 31, DK-2950 Vedbæk (DK). BRANDT, Jakob [DK/DK]; Tjørnevangelen 1, st., DK-2700 Brønshøj (DK). VAD, Knud [DK/DK]; Duevej 112, 2. Tv., DK-2000 Frederiksberg (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: METHOD FOR THE PRODUCTION OF POLYPEPTIDES (57) Abstract <p>The present invention relates to a novel method for the production of short chain polypeptides, including polypeptides having up to 3 disulfide bonds and/or structures rich in basic amino acid residues, and open structured short chain polypeptides, e.g. glucagon, glucagon like peptides and their functional analogues, in genetically modified yeast cells having reduced activity of YAP3 protease. The invention further comprises genetically modified yeast cells, and a method for the preparation of said yeast cells.</p>		

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METHOD FOR THE PRODUCTION OF POLYPEPTIDES

FIELD OF THIS INVENTION

5 The present invention relates to a novel method for the production of short chain polypeptides, including polypeptides having up to 3 disulfide bonds and/or structures rich in basic amino acid residues, and open structured short chain polypeptides, e.g. glucagon, glucagon like peptides and their functional analogues, in genetically modified yeast cells, said genetically modified yeast cells, and a method for the
10 preparation of said yeast cells.

BACKGROUND OF THIS INVENTION

15 Expression of heterologous proteins in yeast after transformation of yeast cells with suitable expression vectors comprising DNA sequences coding for said proteins has been successful for many species of polypeptides, such as glucagon, glucagon like peptides and their functional analogues. Yeasts, and especially *Saccharomyces cerevisiae*, are preferred host microorganisms for the production of pharmaceutically
20 valuable polypeptides due to the stable yield and safety.

However, it is often found that the expression product is a heterogeneous mixture of species of the desired polypeptide precursor having different amino acid chain lengths. A number of proteases, activated by the PEP4 gene product are responsible for yeast
25 protein degradation. Mutation in the PEP4 gene such as the pep4-3 mutation is often used to reduce cellular proteolysis whereby the quality and yields of heterologous proteins of interest can be improved. EP 341215 describes the use of a yeast strain that lacks carboxypeptidase ysc α activity for the expression of the heterologous protein hirudin. Wild-type yeast strains produce a mixture of desulphatohirudin species
30 differing in the C-terminal sequence due to the post-translational action of endogeneous yeast proteases on the primary expression product. It is shown that

yeast mutant strains lacking carboxypeptidase $ysc\alpha$ activity are unable to remove amino acids from the C-terminus of heterologous proteins and therefore give rise to integral proteins.

The use of yeast strains defective in protease A, B, Y, and/or S activity can only
5 partially reduce random proteolysis of foreign gene products.

Another problem encountered in production of heterologous proteins in yeast is low yield, presumably due to proteolytic processing both in intracellular compartments and at the plasma membrane caused by aberrant processing at internal sites in the protein
10 e.g. secretion of human parathyroid hormone (Gabrielsen et al. Gene 90: 255-262, 1990; Rokkones et al. J. Biotechnol. 33: 293-306, 1994), and secretion of β -endorphine by *S. cerevisiae* (Bitter et al. Proc. Natl. Acad. Sci. USA 81: 5330-5334, 1984). Some polypeptides, e.g. polypeptides having from about 10 to about 55 amino acids or shorter chains and none or a few disulphide bonds and/or are rich in basic
15 amino acids, such as β -endorphine, glucagon and glucagon like peptides may be especially susceptible to intracellular and extracellular proteolytic degradation when expressed in a heterologous host due to their short-chain open and non-stable structure resulting in an inhomogeneous product.

20 WO 95/23857 discloses production of recombinant human albumin (rHA), which is a very large carrier-type protein cross-linked with 17 disulphide bonds and having a molecular weight of about 66 kD, in yeast cells having a reduced level of yeast aspartyl protease 3 (Yap3p) proteolytic activity resulting in a reduction of undesired 45 kD rHA fragment and in a 30 to 50% increased yield of recovered rHA produced by the haploid
25 $\Delta yap3$ yeast strain compared to the rHA produced by the corresponding haploid YAP3 wild-type yeast strain.

Previously, Bourbonnais et al. (Biochimie 76: 226-233, 1994), have shown that the YAP3 protease gene product has in vitro substrate specificity which is distinct though
30 overlapping with the Kex2p substrate specificity, and shown that Yap3p cleaves exclusively C-terminal to arginine residues present in the prosomatostatin's putative

processing sites. Moreover, Cawley et al. (J. Biol. Chem. 271: 4168-4176, 1996) have determined the in vitro specificity and relative efficiency of cleavage of mono- and paired-basic residue processing sites by Yap3p for a number of prohormone substrates, such as bovine proinsulin.

5

The purpose of the present invention is to provide an improved method for the production of secreted polypeptides having up to about 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40 or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no
10 more than one disulphide bond, in the structure in a yeast expression system. Preferred examples of polypeptides are glucagon and glucagon like peptides, CRF, and truncated and/or C-or N-terminally truncated and/or N-terminally extended forms of cocaine amphetamine regulated transcript (CART). Preferably, the production of polypeptides according to the invention is considerably increased, e. g. more than two
15 fold compared to the production of said polypeptides in conventional yeast expression systems.

Often it is advantageous to produce heterologous polypeptides in a diploid yeast culture, because possible genetical defects may become phenotypically expressed in a
20 haploid yeast culture, e.g. during continuous fermentation in production scale, and because the yield may be higher (Fu et al. Biotechnol. Prog., 12: 145-148, 1996; Mead et al. Biotechnol. Letters, 8: 391-396, 1986).

It would be obvious for a person skilled in the art to use the method of the present
25 invention to produce other polypeptides satisfying the above criteria, such as insulin and insulin analogues, adrenocorticotrophic hormones, angiotensinogen, atrial natriuretic peptides, dynorphin, endorphines, galanin, gastrin, gastrin releasing peptides, neuropeptide Y fragments, pancreastatin, pancreatic polypeptides, secretin, vasoactive intestinal peptide, growth hormone releasing factor, melanocyte stimulating
30 hormone, neurotensin, adrenal peptide, parathyroid hormone and related peptides, somatostatin and related peptides, brain natriuretic peptide, calcitonin, corticotropin

releasing factor (CRF), cf. SEQ ID NO: 3 herein, thymosin, and urotensin; and homologous or otherwise related peptides and fragments of these and other polypeptides (e.g. EEID-CART₅₅₋₁₀₂, cf. SEQ ID NO: 2 herein), as long as the criteria of having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40 or from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, is fulfilled.

SUMMARY OF THE INVENTION

10

The above identified purpose is achieved with the method according to the present invention which comprises culturing a yeast which has reduced activity of Yap3 protease (Yap3p) or a homologue thereof and has been transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40, or from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, such as glucagon or glucagon like peptides, and isolating said polypeptides. Preferably, the yeast cells lack Yap3p activity through disruption of the YAP3 gene.

20

Using a YAP3 disrupted yeast strain for the production of polypeptides having from 1-70 amino acids, preferably from 1-40, and more preferably from 10-30 amino acids in the polypeptide chain, and having no more than one disulphide bonds in the structure such as polypeptides encoded by the glucagon precursor gene including glucagon, GRPP, GLP-1, GLP-2, and their functional analogues thereof result in a remarkably improved yield of up to about 2-fold and even 10-fold compared to the yield from the corresponding YAP3 wild-type yeast strain. It has been found that using a YAP3 disrupted yeast strain for the production of heterologous polypeptides having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40 or from 25-35 amino acids, in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, such as polypeptides

30

encoded by the glucagon precursor gene including glucagon, GRPP, GLP-1, GLP-2, and their functional analogues thereof or CRF, e.g. as shown in SEQ ID NO:3 herein, or truncated and/or N-terminally extended forms of CART, preferably EEID-CART₅₅₋₁₀₂ as shown in SEQ ID NO:2 herein, result in a remarkably improved yield of the
5 heterologous polypeptide of up to about 2-fold and even 10-fold compared to the yield obtained from the corresponding YAP3 wild-type yeast strain. Another advantage of using the method of the invention for production of heterologous polypeptides is that the secreted product has an improved homogeneity due to a reduced degree of proteolytic degradation.

10

The present inventors have also found that the use of a diploid YAP3 disrupted yeast in the method of the invention results in a significantly higher production level of secreted heterologous polypeptide which is about 2-fold and even 9-fold higher compared to the yield level from the corresponding wild-type haploid yeast.

15

Suitably, the yeast is *S. cerevisiae* which lacks a functional YAP3 gene. However, other yeast genera may have equivalent proteases, i.e. homologues of Yap3p, e. g. the genera *Pichia* and *Kluyveromyces* as shown in WO 95/23857 and Clerc et al. (*J. Chromat. B.* 662: 245-259, 1994). A gene is regarded as a homologue, in general, if
20 the sequence of the translation product has greater than 50% sequence identity to Yap3p. Komano and Fuller (*Proc. Natl. Acad. Sci. USA* 92: 10752-10756, 1995) has identified the Mkc7 aspartyl protease from *S. cerevisiae* which is closely related to Yap3p (53% identity). Other aspartyl proteases of *Saccharomyces* include the gene products of PEP4, BAR1, and of open reading frames, the sequences of which are
25 partially homologous with the YAP3 open reading frame, such as YAP3-link (coded by GenBank acc. No. X89514: pos. 25352-26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116). According to recently accepted yeast genome nomenclature the yeast gene names YAP3, YAP3 link, YIV 9, NO 4, and MKC 7 used herein correspond to the yeast open
30 reading frame YLR120C, YLR121C, YIR039C, YDR349C, and YDR144C,

respectively. Furthermore, the gene product of open reading frame YGL259W is included among the yeast aspartyl proteases.

Examples of yeasts include *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*,
5 *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida sp.*, *Candida utilis*, *Candida cacaoi*, *Geotrichum sp.*, and *Geotrichum fermentans*.

A suitable means of eliminating the activity of a protease is to disrupt the host gene
10 encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease including regulatory and/or coding regions, or, alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations. Other methods which may be suitable for down regulation of the protease include the introduction of antisense and/or
15 ribozyme constructs in the yeast, e.g. Atkins et al. (Antisense and Development 5: 295-305, 1995) and Nasr et al. (Mol. Gen Genet 249: 51-57, 1995). One preferred method of disrupting the YAP3 gene in the yeast strain used in the method of the present invention are described by Rothstein (Method in Enzymol, 194: 281-301, 1991).

20

The expression "glucagon or glucagon like peptides" as used herein may be of human origin or from other animals and recombinant or semisynthetic sources and include all members of the glucagon family, such as GRPP (glicentine related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2),
25 including truncated and/or N-terminally extended forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y, GLP-2 A19T+34Y, GLP-2 A2G and GLP-2 A19T, and other analogues having from 1 to 3 amino acid changes, additions and/or deletions. The cDNA used for expression of the polypeptide according to the invention include codon optimised forms for expression in yeast.

30

Throughout the description and claims is used one and three letter codes for amino acids in accordance with the rules approved (1974) by the IUPAC-IUB Commission on Biochemical Nomenclature, vide Collected Tentative Rules & Recommendations of the Commission on Biochemical Nomenclature IUPAC-IUB, 2nd ed., Maryland, 1975.

5

A further aspect of the invention is a culture of yeast cells transformed with a hybrid vector containing a polynucleotide sequence, preferably a DNA sequence, encoding a polypeptide having up to 55 amino acids, preferably from 10 to 50 amino acids, more preferably from 15 to 40, or preferably from 20 to 30 amino acids, most preferably from
10 25 to 35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, said polynucleotide sequence or DNA sequence being operably linked to a polynucleotide sequence or DNA sequence encoding a yeast promoter and a leader sequence (pro sequence or prepro sequence) and/or other polynucleotide sequences or DNA
15 sequences that are necessary for said polypeptide to be expressed in and secreted from the yeast, said culture of yeast cells being characterized in that the cells have reduced Yap3p activity, preferably through a disruption of the YAP3 gene, and said culture of yeast cells being a culture of haploid or polyploid, preferably diploid, yeast cells.

20

In another aspect the invention provides a culture of yeast cells containing a polynucleotide sequence encoding a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40 or preferably from 20-30 amino acids, most preferably from 25 to 35 amino acids, and having from 0 to 3
25 disulphide bonds, preferably one or less disulphide bonds in the structure, and a second polynucleotide sequence encoding a secretion signal causing said polypeptide to be expressed in and secreted from the yeast, characterized in that the yeast cells have reduced Yap3 protease activity. Preferably, the yeast cells are diploid yeast cells transformed with a hybrid vector comprising said polynucleotide sequences, and
30 preferably the yeast cells lack Yap3p activity which may conveniently be obtained through disruption of the YAP3 gene.

- The DNA encoding the polypeptides having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bonds in the structure, may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration on host chromosome(s) is desired.
- Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.
- If integration is desired, the DNA is inserted into an yeast integration plasmid vector, such as pJJ215, pJJ250, pJJ236, pJJ248, pJJ242 (Jones & Prakash, Yeast 6: 363, 1990) or pDP6 (Fleig et al. Gene 46:237, 1986), in proper orientation and correct reading frame for expression, which is flanked with homologous sequences of any non-essential yeast genes, transposon sequence or ribosomal genes. Preferably the flanking sequences are yeast protease genes or genes used as a selective marker. The DNA is then integrated on host chromosome(s) by homologous recombination occurred in the flanking sequences, by using standard techniques shown in Rothstein (Method in Enzymol, 194: 281-301, 1991) and Cregg et al. (Bio/Technol. 11:905-910, 1993).
- Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the polypeptides to be produced according to the method of the invention, preferred examples of polypeptides being glucagon, glucagon like peptides, CRF and

EEID-CART₅₅₋₁₀₂, or their functional analogues, which can then be recovered, as is known.

Useful yeast plasmid vectors include the POT (Kjeldsen et al. Gene 170: 107-112, 1996) and YEp13, YEp24 (Rose and Broach, Methods in Enzymol. 185: 234-279, 1990), and pG plasmids (Schena et al. Methods in Enzymol. 194: 289-398, 1991).

Methods for the transformation of *S. cerevisiae* include the spheroplast transformation, lithium acetate transformation, and electroporation, cf. Methods in Enzymol. Vol. 194 (1991). Preferably the transformation is as described in the examples herein.

Suitable promoters for *S. cerevisiae* include the MF α 1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CYC1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoter. A suitable promoter in the genus *Pichia* is the AOX1 (methanol utilisation) promoter.

The transcription terminal signal is preferably the 3' flanking sequence of a eucaryotic gene which contains proper signal for transcription termination and polyadenylation. Suitable 3' flanking sequences may, e.g. be those of the gene naturally linked to the expression control sequence used, i.e. corresponding to the promoter.

The DNA constructs that are used for providing secretory expression of the polypeptide according to the invention comprise a DNA sequence that includes a leader sequence linked to the polypeptide by a yeast processing signal. The leader sequence contains a signal peptide ("pre-sequence") for protein translocation across the endoplasmic reticulum and optionally contains an additional sequence ("pro-sequence"), which may or may not be cleaved within yeast cells before the polypeptide is released into the surrounding medium. Useful leaders are the signal peptide of mouse α -amylase, *S. cerevisiae* MF α 1, YAP3, BAR1, HSP150 and *S. kluyveri* MF α signal peptides and prepro-sequences of *S. cerevisiae* MF α 1, YAP3, PRC, HSP150,

and S. kluyveri MF α and synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666. Furthermore, the polypeptides to be produced according to the method of the invention may be provided with an N-terminal extension as described in WO 95/35384.

5

The invention also relates to a method of preparing a yeast having reduced Yap3p activity comprising the steps of a) providing a hybrid plasmid containing a part of the YAP3 gene and suitable for transformation into a yeast cell, b) disrupting the YAP3 gene by deleting the fragment of YAP3 and inserting the URA3 gene instead to obtain
10 a $\Delta yap3::URA3$ gene disruption plasmid, c) providing a yeast $\Delta ura3$ deletion mutant, d) transforming said mutant with said plasmid, and e) selecting the $\Delta yap3::URA3$ deletion mutants on a medium without uracil. Further the invention relates to a method of preparing a yeast having reduced Yap3p activity using antisense technology.

15 Moreover, the polypeptides to be produced according to the method of the invention may conveniently be expressed coupled to an N- or C-terminal tag or as a precursor or fusion protein although the total length of the expressed polypeptide may exceed a total of 55 or 70 amino acids.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the construction of the pS194 plasmid.

Fig. 2 shows the construction of plasmids pME834 and pME1389.

Fig. 3 is a restriction map of the human glucagon expression plasmid pMT703.

25 Fig. 4 is a restriction map of the human GLP-1(7-37) expression plasmid pLaC253.

Fig. 5 is a restriction map of the human GLP-2 expression plasmid pKV210.

Fig. 6 is a restriction map of the pME973 plasmid, containing the genes encoding the HO (homothallism) endonuclease and Ura3p inserted into the YEpl3 plasmid.

30

DETAILED DESCRIPTION OF THIS INVENTION

Preferred embodiments of this invention are described in Table 1 below. Having knowledge of the art, it will be obvious to a skilled person to produce other polypeptides having up to 55 amino acids, preferably from 10-50 amino acids, preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure and their functional analogues by the method of the present invention using similar constructs.

10

Table 1

Presequence (signal)	Prosequence	Heterologous protein
MF α 1 (1-19)	MF α 1(20-85)	Glucagon
MF α 1 (1-19)	MF α 1(20-81)MAKR	DDDDK-Glucagon
MF α 1 (1-19)	MF α 1(20-85)	GLP-1(7-37)
YAP3(1-21)	LA19 ¹ KR	GLP-1(7-35)R36A
spx3 ²	LaC212	GRPP
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2
HSP150(1-18)	HSP150(19-67)- WIIAENTTLANVAMAKR	GLP-2
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue F22Y
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue A19T, +34Y
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue A19T
MF α 1 (1-19)	MF α 1(20-81)MAKR	GLP-2 analogue A2G
MF α 1 (1-19)	MF α 1(20-81)MAXKR X= peptide bond or Y or S or K or E or ARS	Glucagon or Calcitonin

¹ LA19, cf. SEQ ID NO:1 herein and WO 95/34666, ² spx3-LaC212, cf. WO 89/02463 and WO 90/10075.

15

The Genetic background of *S. cerevisiae* strains used herein is as follows:

E11-3C	MAT α YAP3 pep4-3 Δ tpi::LEU2 leu2 URA3
5 SY107	MAT α YAP3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
ME1487	MAT α Δ yap3::URA3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
ME1656	MAT α Δ yap3::ura3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
10 ME1684	MAT α Δ yap3::URA3:: Δ ylr121c pep4-3 Δ tpi::LEU2 leu2 Δ ura3
ME1695	MAT α Δ yap3::ura3 pep4-3 Δ tpi::LEU2 leu2 Δ ura3
15 ME1719	MAT α / α Δ yap3::URA3/ Δ yap3::ura3 pep4-3/pep4-3 Δ tpi::LEU2/ Δ tpi::LEU2 leu2/leu2 Δ ura3/ Δ ura3
20 MT663	MAT α / α YAP3/YAP3 pep4-3/pep4-3 Δ tpi::LEU2/ Δ tpi::LEU2 leu2/leu2 URA3/URA3 HIS4/his4

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the fore-going description and in the following examples may, both separately and in
 25 any combination thereof, be material for realizing the invention in diverse forms thereof.

Example 1

30 Δ yap3::URA3 gene disruption

The Δ ura3 deletion mutation was constructed as follows:
 pJJ244 (pUC18 containing a 1.2 kb HindIII fragment of the URA3 gene) was digested with Styl and filled in with Klenow polymerase and self ligated. The resulting
 35 plasmid designated pS194 contains a 84bp of Styl-Styl fragment deletion of the URA3 gene, cf. Fig.1.

The $\Delta yap3::URA3$ gene disruption plasmid pME1389 was constructed as follows:

The 2.6kb *SacI*-*PstI* fragment which contains the YAP3 gene in pME768 (Egel-Mitani et al. Yeast 6: 127-137, 1990) was inserted in 2.6 kb of the *SacI*-*PstI* fragment of pIC19R (Marsh et al. Gene 32: 481-485, 1984). The resulting plasmid is pME834.

- 5 pME834 was digested with *HindIII* to form a deletion of the 0.7 kb YAP3 fragment and the 1.2 kb *HindIII* fragment of the URA3 gene (Rose et al. Gene 29: 113-124, 1984) was inserted instead. The resulting plasmid is pME1389. The construction of plasmids pME834 and pME1389 is shown in Fig. 2 in diagrammatic form.

- 10 *S. cerevisiae* strain E11-3C ($MAT\alpha$ YAP3 pep4-3 $\Delta tpi::LEU2$ leu2 URA3), cf. ATCC 20727, US pat. 4766073, was transformed with linearized pS194 (*BsgI* digested) to make Δura deletion mutation. By selection on 5-FOA (5-fluoro-orotic acid) containing minimal plates, the $\Delta ura3$ mutant designated SY107 was obtained.

- 15 The strain SY107 ($MAT\alpha$ YAP3 pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$), was then transformed with pME1389 previously being cut by *Sall* and *SacI*, and 3kb fragment of $\Delta yap3::URA3$ was isolated for the transformation. $\Delta yap3::URA3$ deletion mutants were selected on minimal plates without uracil. URA3 transformants were characterized by PCR and Southern hybridisation to confirm the correct integration of
- 20 the $\Delta yap3::URA3$ fragment in the YAP3 locus. ME1487 was isolated as a $\Delta yap3::URA3$ deletion mutant ($MAT\alpha$ $\Delta yap3::URA3$ pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$).

Example 2

25

Construction of a diploid $\Delta yap3/\Delta yap3$ strain

- ME1487 was mutagenized by using EMS (methane-sulfonic acid ethylester) and *ura3* mutants were selected on plates containing 5-FOA. One of the selected
- 30 isolates, ME1656 was then subjected to mating type switch (Herskowitz and Jensen, Methods in Enzymol. 194: 132-146, 1991) by transient transformation with pME973

shown in Fig. 6. pME973 contains the genes encoding the HO (homothallism) endonuclease and URA3 inserted into the YEp13 plasmid (Rose and Broach, Methods in Enzymol. 185: 234-279, 1990). From transient transformants, ME1695 was selected as the haploid strain, which had switched from MAT α to MATa, and
5 have the following genetic background: MATa $\Delta yap3::ura3$ pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$.

ME1695 was then crossed with ME1487 by micromanipulation (Sherman and Hicks, Methods in Enzymol. 194: 21-37, 1991) in order to get $\Delta yap3/\Delta yap3$ diploids. From
10 the resulting diploids, ME1719 was selected as the strain with the following genetic background:
MATa/ α $\Delta yap3::ura3/\Delta yap3::URA3$ pep4-3/pep4-3 $\Delta tpi::LEU2/\Delta tpi::LEU2$ leu2/leu2 $\Delta ura3/\Delta ura3$.

15

Example 3

Construction of a $\Delta yap3::URA3::\Delta ylr121c$ double disruption strain

20 In order to make a one-step gene disruption strain of the two closely linked genes encoding YAP3 and YLR121C, the following two oligonucleotide primers were synthesized:

P1 Length 57bp: YLR121C/URA3 primer

25

5'-GAT CGA ACG GCC ATG AAA AAT TTG TAC TAG CTA ACG
AGC AAA GCT TTT CAA TTC AAT-3'

P2 Length 57bp: YAP3/URA3 primer

30

5'-CCA GAA TTT TTC AAT ACA ATG GGG AAG TTG TCG TAT
TTA TAA GCT TTT TCT TTC CAA-3'

P1 and P2 each contains 40 nucleotides corresponding to sequences within the
5 coding region of YLR121C and YAP3, respectively, as well as a HindIII site
(AAGCTT) and 12 nucleotides corresponding to sequences flanking the URA3 gene
(YEL021W). P1 and P2 were used for PCR using the URA3 gene as template. The
resulting 1248bp PCR fragment contains the URA3 selective marker flanked with 40
nucleotides derived from the YAP3 or YLR121C encoding regions. The PCR
10 fragment was then transformed into ME1655, and $\Delta yap3::URA3::\Delta ylr121c$ deletion
mutants were selected and characterized as described in Example 1. ME1684 was
isolated as a $\Delta yap3::URA3::\Delta ylr121c$ mutant with the following genetic background:
MAT α $\Delta yap3::URA3::\Delta ylr121c$ pep4-3 $\Delta tpi::LEU2$ leu2 $\Delta ura3$.

15 Example 4

Transformation into yeast

In order to make yeast competent cells, yeast haploid strains SY107 and ME1487 or
20 the diploid ME1719 strain were cultivated in 100ml YPGGE medium (1% yeast
extract, 2% peptone, 2% glycerol, 2% galactose, 1% ethanol) to OD₆₀₀ = 0.2. Cells
were harvested by centrifugation at 2000rpm for 5 min. and washed once by 10ml
H₂O. Cells were resuspended in 10ml SED (1M sorbitol, 25mM Na₂EDTA pH8,
6.7mg/ml dithiothreitol) and incubated at 30°C for 15min. Cells were harvested by
25 centrifugation and resuspended in 10ml SCE (1M sorbitol, 0.1M Na-citrate, 10mM
Na₂EDTA, pH5.8) + 2mg Novozyme SP234 and incubated at 30°C for 30 min. After
cells were harvested by centrifugation and washed once by 10ml 1.2M sorbitol and
subsequently by 10ml CAS (1M sorbitol 10mM CaCl₂, 10mM Tris-HCl pH7.5), cells
were harvested by centrifugation and resuspended finally in 2ml CAS. Competent
30 cells were frozen in portion of 100 μ l per Eppendorf tube at -80°C.

Transformation was made as follows: Frozen competent cells (100 μ l) were warmed up quickly and 1 μ g plasmid DNA were added. Cells were incubated at room temp. for 15 min. and 1ml PEG solution (20% polyethyleneglycol 4000, 10mM CaCl₂, 10mM Tris-HCl pH7.5) was added. After 30min. at room temperature, cells were

5 harvested by centrifugation at 2000rpm for 15min. and resuspended in 100 μ l SOS (1M sorbitol, 1/2 vol. YPGGE, 0.01% uracil, 7mM CaCl₂). After incubating at 30°C for 2 hours, cells were centrifuged and resuspended in 0.5ml 1M sorbitol. Cells were then spread on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) together with 6ml of top agar (YPD containing 2.5% agar). Plates were incubated at

10 30°C for 3 to 5 days until transformants appear.

Example 5

Heterologous protein expression plasmid

15 Yeast-E.coli shuttle vector used in the following examples contains a heterologous protein expression cassette, which includes a DNA sequence encoding a leader sequence followed by the heterologous polypeptide in question operably placed in between the TPI promoter and TPI terminator of *S. cerevisiae* in a POT plasmid

20 (Kjeldsen et al. 1996, *op. cit.*). The leader sequences are the MF α 1 prepro-sequence and modification thereof. Examples are shown as follows:

Table 2

Presequence (signal)	Presequence	Heterologous protein	plasmid
MF α 1(1-19)	MF α 1(20-85)	Glucagon	pMT703 Fig. 3
MF α 1(1-19)	MF α 1(20-85)	GLP-1(7-37)	pLaC253 Fig. 4
MF α 1(1-19)	MF α 1(20-81)MAKR	GLP-2	pKV210 Fig. 5
MF α 1(1-19)	MF α 1(20-81)MAKR	CRF ₁₋₄₁	pKV241
MF α 1(1-19)	MF α 1(20-81)MAKR	EEID-CART ₅₅₋₁₀₂	pSX647

Example 6

5 Expression of glucagon

Human glucagon expression plasmid pMT703, cf. Figure 3, was transformed into three strains, such as, YAP3 disrupted haploid strain ME1487($\Delta yap3$), YAP3 disrupted diploid strain ME1719 ($\Delta yap3/\Delta yap3$) and YAP3 wild-type strain SY107 (YAP3 WT). Transformants were selected by glucose utilization as a carbon source in YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% glucose, 2% agar). ME1532 and YES1746 are pMT703 transformants obtained from ME1487 ($\Delta yap3$) and ME1719 ($\Delta yap3/\Delta yap3$), respectively, whereas ME1530 is the pMT703 transformant obtained from SY107 (YAP3 WT). Transformants were cultivated in 5ml YPD liquid medium at 30°C for 3 days with shaking at 200rpm. Culture supernatants were obtained after centrifugation at 2500rpm for 5 min. and 1ml supernatants were analyzed by reverse phase HPLC. Production levels, shown in Table 3, were average value of cultures from 2 independently isolated transformants (Exp.1) or values from a mixculture of 3 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses showed that ME1532 or YES1746 produced approx. 4 to 6 times more glucagon than ME1530.

HPLC setting for glucagon detection

25 HPLC-Column: 4 x 250 mm Novo Nordisk YMC-OdDMeSi C18 5 μ m
Column temp.: 50°C
Flowrate: 1 ml/min
30 HPLC solvents:
A: 10 % (v/v) acetonitrile in 0.2 M Na_2SO_4 , 0.04 M
 H_3PO_4 pH adjusted to 2.3 with ethanolamine

B: 50 % (v/v) acetonitrile in water

Inj. vol: 150 μ l

Glucagon was eluted from the HPLC columns with 23.6 % acetonitrile to 32.9 % acetonitrile in 40 min.

Table 3

TRANSFORMANT	HOST	PLASMID	GLUCAGON LEVEL	
			Exp. 1	Exp. 2
ME1530	SY107	pMT703	100%	100%
ME1532	ME1487	pMT703	596%	469%
YES1746	ME1719	pMT703	ND	587%

Example 7

Expression of GLP-1(7-37)

Human GLP-1(7-37) expression plasmid pLaC253, cf. Figure 4, was transformed into ME1487(Δ yap3), ME1719 (Δ yap3/ Δ yap3) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1535 and YES1823 are the pLaC253 transformants obtained from ME1487(Δ yap3) and ME1719 (Δ yap3/ Δ yap3), respectively, whereas ME1534 is the pLaC253 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 4, were average value of cultures from 2 independently isolated transformants (Exp.1) or values from a mixture of 3 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses showed that ME1535 or YES1823 produced approx. 2 to 3 times more GLP-1(7-37) than ME1530.

HPLC settings for GLP-1(7-37) detection:

As described in Example 6, except that GLP-1 was eluted from HPLC columns with 31.2% acetonitrile to 41.2% acetonitrile in 40 min.

5

Table 4

TRANSFORMANT	HOST	PLASMID	GLP-1(7-37) LEVEL	
			Exp. 1	Exp. 2
ME1534	SY107	pLaC253	100%	100%
ME1535	ME1487	pLaC253	287%	
YES1823	ME1719	pLaC253	ND	161%

10

Example 8**Expression of GLP-2**

Human GLP-2 expression plasmid pKV210, cf. Figure 5, was transformed into ME1487 ($\Delta yap3$), ME1719 ($\Delta yap3/\Delta yap3$) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1615 and YES1827 are the pKV210 transformants obtained from ME1487 ($\Delta yap3$) and ME1719 ($\Delta yap3/\Delta yap3$), respectively, whereas ME1614 is the pKV210 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 5 were average value of cultures from 2 independently isolated transformants (Exp.1) or values from a mixture of 3 transformants (Exp.2), and were normalized so that the YAP3 wild-type level was taken as 100%. HPLC analyses showed that ME1615 and YES1827 produced approx. 6 to 11 times more GLP-2 than ME1614.

25

HPLC settings for GLP-2 detection:

HPLC-Column: Vydac 214TP54 Column

Flowrate: 1 ml/min

30

HPLC solvents:

A: 0.1% TFA
B: 0.07% TFA in acetonitrile

5

GLP-2 was eluted from the HPLC columns with 0.07% TFA in 20% to 80% acetonitrile in 60 min.

10

Table 5

TRANSFORMANT	HOST	PLASMID	GLP-2 LEVEL	
			Exp. 1	Exp. 2
ME1614	SY107	pLaC210	100%	100%
ME1615	ME1487	pLaC210	1130%	682%
YES1827	ME1719	pLaC210	ND	675%

Example 9

15 **Expression of CRF₁₋₄₁ (SEQ ID NO:3 herein)**

Human Corticotropin Releasing Factor (CRF₁₋₄₁) expression plasmid pKV241 (equivalent to pKV210 Fig. 5 in which MF α 1-pre-pro(1-81)MAKR-GLP2 is substituted by MF α 1-pre-pro(1-81)MAKR-CRF₁₋₄₁) was transformed into
20 ME1487(Δ yap3), ME1719 (Δ yap3/ Δ yap3) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1813 and YES1810 are the pKV241 transformants obtained from ME1487(Δ yap3) and ME1719 (Δ yap3/ Δ yap3), respectively, whereas ME1812 is the pKV241 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 6, were values from a
25 mixculture of 3 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses were performed as in example 8 and showed that ME1813 or YES1810 produced approx. 8 to 9 times more CRF₁₋₄₁ than ME1812.

30 **Table 6**

TRANSFORMANT	HOST	PLASMID	CRF ₁₋₄ LEVEL	
			Exp. 1	Exp. 2
ME1812	SY107	pKV241	ND	100%
ME1813	ME1487	pKV241	ND	834%
YES1810	ME1719	pKV241	ND	911%

Example 10

Expression of EEID-CART₅₅₋₁₀₂ (SEQ ID NO:2 herein)

5 N-terminal extended (EEID) fragment of Human Cocaine and Amphetamine regulated transcript (EEID-CART₅₅₋₁₀₂) expression plasmid pSX637 (equivalent to pKV210 Fig. 5, in which MF α 1-pre-pro(1-81)MAKR-GLP2 is substituted by MF α 1-pre-pro(1-81)MAKR-EEID-CART₅₅₋₁₀₂), was transformed into ME1487(Δ yap3), ME1719 (Δ yap3/ Δ yap3) and SY107 (YAP3 WT). Transformants were selected and analysed as described in Example 6. ME1817 and YES1820 are the pSX637 transformants obtained from ME1487(Δ yap3) and ME1719 (Δ yap3/ Δ yap3), respectively, whereas ME1816 is the pSX637 transformant obtained from SY107 (YAP3 WT). Production levels, shown in Table 7, were values from a mixculture of 3 transformants (Exp.2), and were normalised so that the haploid YAP3 wild-type level was taken as 100%. HPLC analyses were performed as in example 8 and showed that ME1817 or YES1820 produced approx. 2 to 3 times more EEID-CART₅₅₋₁₀₂ than ME1816.

20 Table 7

TRANSFORMANT	HOST	PLASMID	EEID-CART ₅₅₋₁₀₂ LEVEL	
			Exp. 1	Exp. 2
ME1816	SY107	pSX637	ND	100%
ME1817	ME1487	pSX637	ND	216%
YES1820	ME1719	pSX637	ND	282%

Example 11

Table 8 shows data for expression levels of human glucagon, GLP-1₍₁₋₃₇₎ and GLP-2 in ME1487 ($\Delta yap3$) transformed by expression plasmids with different pre-sequences (leaders). Expression plasmids are as described in Fig. 5 except for the details given in Table 8. Expression yields are normalised so that the yield obtained in MT663 (YAP3/YAP3) transformants is set to 100%.

10 **Table 8**

Presequence (signal)	Prosequence	Heterologous protein	Expression Plasmid	Yeast transformant	Yield % ME1487
MF α 1(1-19)	MF α (20-81)MA KR	Glucagon	pKV 216	ME1652	267
YAP3 (1-21)	LA19-KR	Glucagon	pKV 225	ME1780	333
MF α 1(1-19)	MF α (20-81)MARS KR	Glucagon	pKV 217	ME1691	400
MF α 1(1-19)	MF α (20-81)MARK KR	Glucagon	pKV 223	ME1692	185
MF α 1(1-19)	MF α (20-81)MARE KR	Glucagon	pKV 238	ME1727	167
MF α 1(1-19)	MF α (20-81)MAERLE KR	Glucagon	pKV 237	ME1726	189
MF α 1(1-19)	MF α (20-81)MAKELE KR	Glucagon	pKV 236	ME1725	234
MF α 1(1-19)	MF α (20-81)MA KR	GLP-1 ₍₁₋₃₇₎	pKV 230	ME1718	189
MF α 1(1-19)	MF α (20-81)MA KR	GLP-2 A19T	pKV 219	ME1677	411
MF α 1(1-19)	MF α (20-81)MA KR	GLP-2 A2G	pKV 220	ME1678	360
MF α 1(1-19)	MF α (20-81)MA KR	GLP-2 F22Y	pKV 249	ME1781	280

SEQUENCE LISTING

5 INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:
10 (A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gln Pro Ile Asp Asp Thr Glu Ser Asn Thr Thr Ser Val Asn Leu
1 5 10 15
20 Met Ala Asp Asp Thr Glu Ser Arg Phe Ala Thr Asn Thr Thr Leu
20 25 30
Ala Leu Asp Val Val Asn Leu Ile Ser Met Ala
25 35 40

30

INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:
35 (A) LENGTH: 52 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Glu Ile Asp Ile Pro Ile Tyr Glu Lys Lys Tyr Gly Gln Val
1 5 10 15
45 Pro Met Cys Asp Ala Gly Glu Gln Cys Ala Val Arg Lys Gly Ala
20 25 30

24

Arg Ile Gly Lys Leu Cys Asp Cys Pro Arg Gly Thr Ser Cys Asn
35 40 45

Ser Phe Leu Leu Lys Cys Leu
5 50

INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS:
10 (A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Glu Glu Pro Pro Ile Ser Leu Asp Leu Thr Phe His Leu Leu
20 1 5 10 15
Arg Glu Val Leu Glu Met Ala Arg Ala Glu Gln Leu Ala Gln Gln
20 25 30
25 Ala His Ser Asn Arg Lys Leu Met Glu Ile Ile
35 40

CLAIMS

1. A method for the production of a short chain polypeptide in yeast, comprising culturing a yeast having reduced activity of Yap3 protease or a homologue thereof,
5 said yeast being transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bonds in the structure, and isolating
10 said polypeptides.
2. A method for the production of an open structured short chain polypeptide in yeast, comprising culturing a yeast having reduced activity of Yap3 protease or of a homologue thereof, said yeast being transformed with a hybrid vector comprising a yeast promoter operably linked to a DNA sequence coding for a polypeptide having
15 from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bonds in the structure, and isolating said polypeptides.
3. A method according to claim 1 or 2, wherein the yeast lacks Yap3 protease activity.
4. A method according to any one of claims 1 to 3, wherein the yeast is a diploid yeast.
- 20 5. A method according to any one of claims 1 to 4, wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida* sp., *Candida utilis*, *Candida cacaoi*, *Geotrichum* sp., and *Geotrichum fermentans*.
- 25 6. A method according to claim 5, wherein the yeast is *S. cerevisiae*.
7. A method according to any one of the preceding claims wherein the yeast additionally has reduced protease activity selected from the group of proteases coded for by BAR1, STE13, PRA, PRB, KEX1, PRC, CPS, and the YAP3 homologues MKC7, YAP3-link (coded by GenBank acc. No. X89514: pos.25352-

26878), YIV9 (Swiss Prot acc. No. P40583), and aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116) genes.

8. A method according to any one of claims 1 to 6 wherein the yeast additionally has reduced protease activity selected from the group of proteases coded for by STE13, PRA, PRB, KEX1, and PRC genes.
9. A method according to any one of claims 1 to 6, wherein the yeast additionally has reduced protease activity of the aspartyl protease encoded by the yeast open reading frame YGL259W or selected from the group of serine proteases coded for by the KEX2 gene and the yeast open reading frame YCR045C and YOR003W,
10. A method according to any one of the preceding claims wherein the yeast additionally has the pep4-3 mutation.
11. A method according to any one of the preceding claims, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a pre-sequence (signal) or a prepro-sequence linked in the proper reading frame to a second DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bonds in the structure, and a DNA sequence containing yeast transcription termination signals.
12. A method according to any one of claims 1 to 9, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a pre-sequence (signal) or a prepro-sequence linked in the proper reading frame to a second DNA sequence coding for a polypeptide having from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bonds in the structure, and a DNA sequence containing yeast transcription termination signals.

13. A method according to claim 11 or 12, wherein the yeast promoter is selected from the group consisting of the MF α 1 promoter, CYC1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoters.
14. A method according to claim 11 or 12, wherein the hybrid vector comprises a first DNA sequence encoding a signal peptide of mouse α -amylase, *S. cerevisiae* MF α 1, BAR1, YAP3 and HSP150 and *S. kluyveri* MF α signal peptides or prepro-sequences of *S. cerevisiae* MF α 1, YAP3, PRC, HSP150 and *S. kluyveri* MF α and/or synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666.
15. A method according to any one of the preceding claims wherein the DNA sequence encoding a polypeptide is selected from the group consisting of DNA sequences encoding glucagon family, such as GRPP (glucocorticotrophic hormone related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2), including truncated forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y, GLP-2 A19T+34Y, GLP-2 A2G and GLP-2 A19T, and other analogues having from 1 to 3 amino acid changes, additions and/or deletions.
16. A method according to any one of claims 1 to 14, wherein the DNA sequence encoding a polypeptide is selected from the group of DNA sequences encoding CRF and a truncated and/or N-terminally extended form of CART, preferably EEID-CART₅₅₋₁₀₂.
17. A culture of yeast cells containing a DNA sequence encoding a polypeptide having up to 55 amino acids, preferably from 10 to 50 amino acids, more preferably from 15 to 40, or preferably from 25 to 35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, and a second DNA sequence encoding a leader sequence causing said polypeptide to be secreted from the yeast, characterized in that said culture of yeast cells has reduced Yap3p activity.

18. A culture of yeast cells according to claim 17 which is transformed with a hybrid vector comprising said DNA sequence and said second DNA sequence.
19. A culture of yeast cells containing a DNA sequence encoding a polypeptide having from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bond in the structure, and a second DNA sequence encoding a leader sequence causing said polypeptide to be secreted from the yeast, characterized in that the yeast cells have reduced Yap3p activity.
20. A culture according to claim 19 which is transformed with a hybrid vector comprising said DNA sequence and said second DNA sequence.
21. A culture according to any one of claims 17 to 20, wherein the yeast cells lacks Yap3p activity.
22. A culture according to any one of claims 17 to 21, wherein the yeast cells are diploid.
23. A culture according to any one of claims 17 to 22, wherein the yeast is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida sp.*, *Candida utilis*, *Candida cacaoi*, *Geotrichum sp.*, and *Geotrichum fermentans*.
24. A culture according to the preceding claim, wherein the yeast is *S. cerevisiae*.
25. A culture according to any one of the claims 17 to 24, wherein the yeast cells additionally have reduced protease activity selected from the group of proteases coded for by the BAR1, STE13, PRA, PRB, KEX1, PRC, CPS, and the YAP3 homologues MKC7, YAP3-link (coded by GenBank acc. No.X89514: pos. 25352-26878), YIV9 (Swiss Prot acc. No. P40583), aspartyl protease (IV) (coded by GenBank acc. No. U28372: pos. 326-2116), genes.
26. A culture according to any one of the claims 17 to 24, wherein the yeast cells additionally have reduced protease activity selected from the group of proteases coded for by the STE13, PRA, PRB, KEX1, and PRC genes.

27. A culture according to any one of the claims 17 to 24, wherein the yeast additionally has reduced protease activity of the *aparatyl* protease encoded by the yeast open reading frame YGL259W or selected from the group of serine proteases coded for by the KEX2 gene and the yeast open reading frame YCR045C and YOR003W.
- 5 28. A culture according to any one of claims 17 to 27, wherein the yeast cells additionally have the *pep4-3* mutation.
29. A culture according to any one of claims 17 to 28, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a presequence (signal) or a preprosequence linked in
10 the proper reading frame to a second DNA sequence coding for a polypeptide having up to 55 amino acids, preferably from 10-50 amino acids, more preferably from 15-40, or preferably from 25-35 amino acids in the polypeptide chain, and having from 0 to 3 disulphide bonds, preferably no more than one disulphide bond in the structure, and a DNA sequence containing yeast transcription termination
15 signals.
30. A culture according to any one of claims 17 to 28, wherein the hybrid vector comprises a yeast promoter operably linked to a first DNA sequence encoding a leader sequence being either a presequence (signal) or a preprosequence linked in the proper reading frame to a second DNA sequence coding for a polypeptide
20 having from 10-50 amino acids, preferably from 15-40, and more preferably from 20-30 amino acids in the polypeptide chain, and having no more than one disulphide bond in the structure, and a DNA sequence containing yeast transcription termination signals.
31. A culture according to claim 29 or 30, wherein the yeast promoter is selected from
25 the group consisting of the $MF\alpha 1$ promoter, CYC1 promoter, galactose inducible promoters such as GAL1, GAL7 and GAL10 promoters, glycolytic enzyme promoters including TPI and PGK promoters, TRP1 promoter, CUP1 promoter, PHO5 promoter, ADH1 promoter, and HSP promoters.
32. A culture according to claim 29 or 30, wherein said first DNA sequence is a signal
30 peptide of mouse α -amylase, *S. cerevisiae* $MF\alpha 1$, BAR1, YAP3 and HSP150 and S.

kluyveri MF α signal peptides or prepro-sequences of *S. cerevisiae* MF α 1, YAP3, PRC, HSP150 and *S. kluyveri* MF α and/or synthetic leader sequences described in WO 92/11378, WO 90/10075 and WO 95/34666.

- 5 33. A culture according to any one of claims 17 to 32, wherein the DNA sequence encoding a polypeptide is a DNA sequence encoding a polypeptide of the glucagon family, such as GRPP (glicentine related polypeptide), glucagon, GLP-1 (glucagon like peptide 1), and GLP-2 (glucagon like peptide 2), including truncated forms, such as GLP-1(7-36), and includes analogues, such as GLP-1(7-35)R36A GLP-2 F22Y, GLP-2 A19T+34Y, GLP-2 A2G and GLP-2 A19T, and other analogues having from
10 1 to 3 amino acid changes, additions and/or deletions.
34. A culture according to any one of claims 17 to 32, wherein the DNA sequence encoding a polypeptide is selected from the group of DNA sequences encoding CRF and a truncated and/or N-terminally extended CART, preferably EEID-CART₅₅.
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- 15 35. A method of preparing a yeast having reduced Yap3p activity comprising the steps of a) providing a hybrid plasmid containing the a part of the YAP3 gene and suitable for transformation into a yeast cell, b) disrupting the YAP3 gene by deleting the fragment of YAP3 and inserting a URA3 gene instead to obtain a $\Delta yap3::URA3$ gene disruption plasmid, c) providing a yeast $\Delta ura3$ deletion mutant, d) transforming
20 said mutant with said plasmid, and e) selecting $\Delta yap3::URA3$ deletion mutants on a medium without uracil.
36. A method of preparing a yeast having reduced Yap3p activity using antisense technology.
37. A method according to claims 35 or 36 wherein the yeast is *S. cerevisiae*.
- 25 38. Any novel feature or combination of features described herein.

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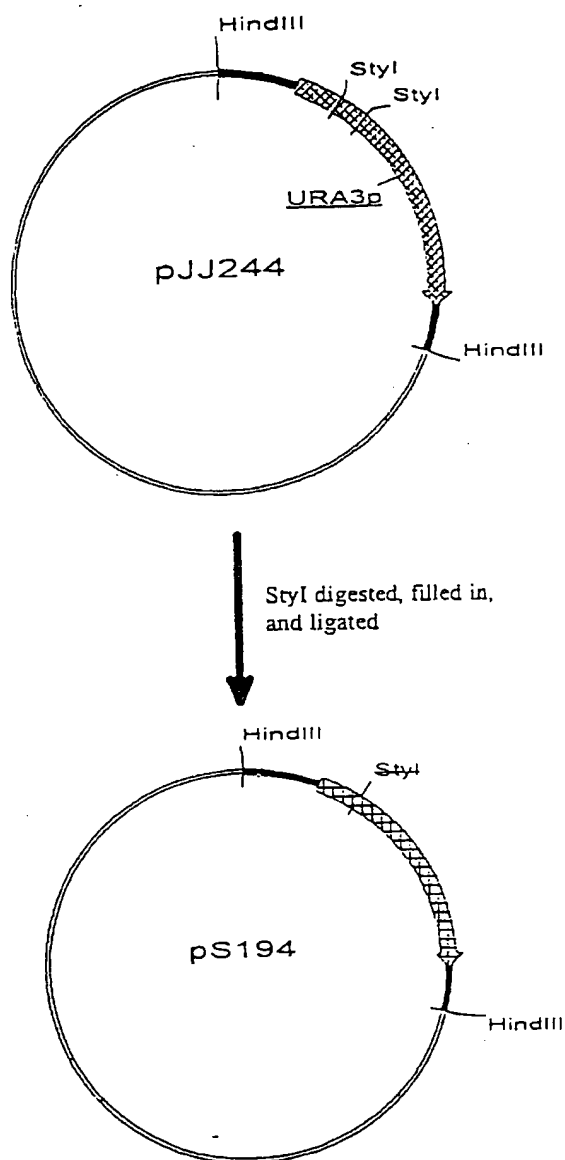


Fig. 1

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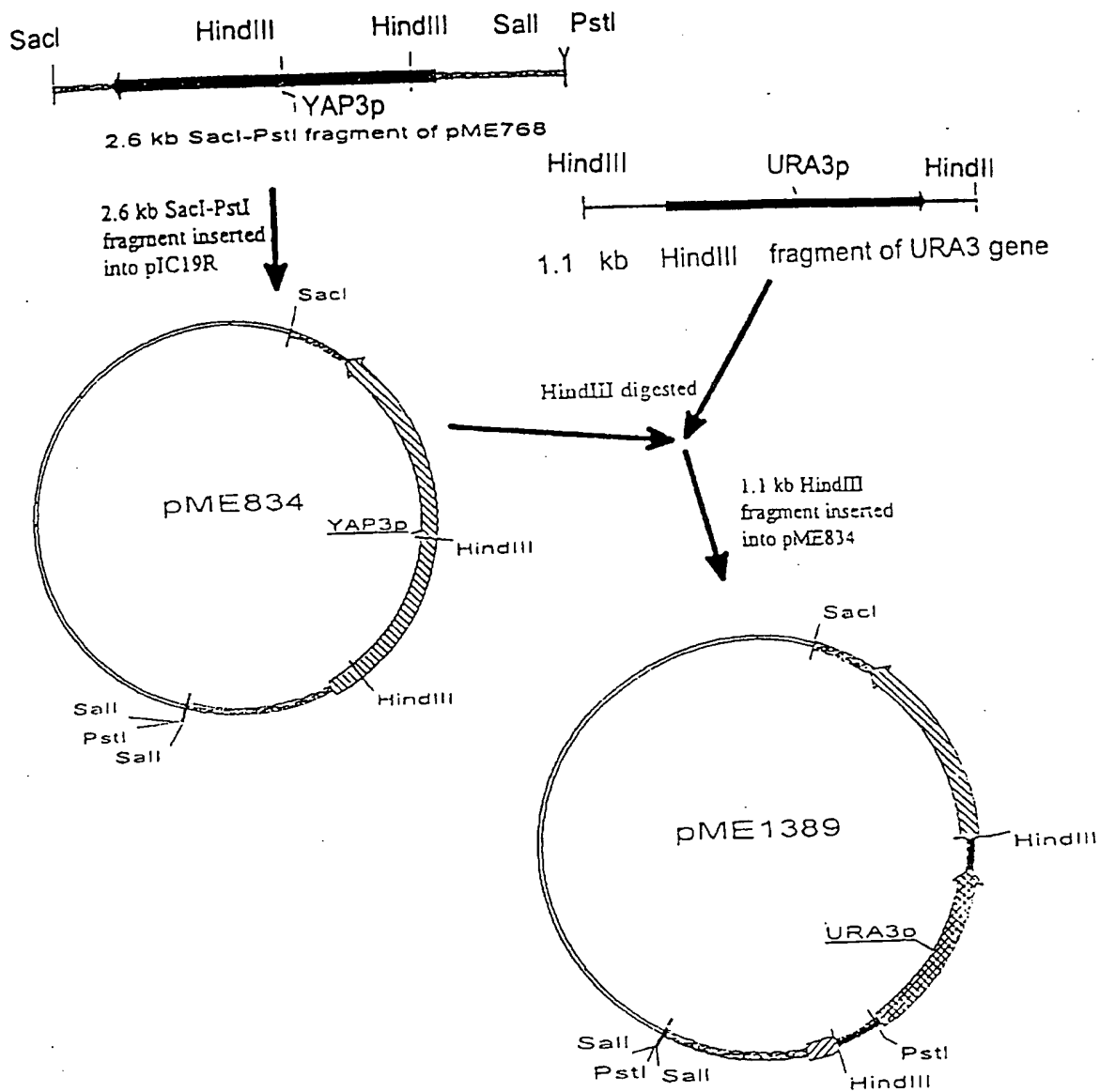


Fig. 2

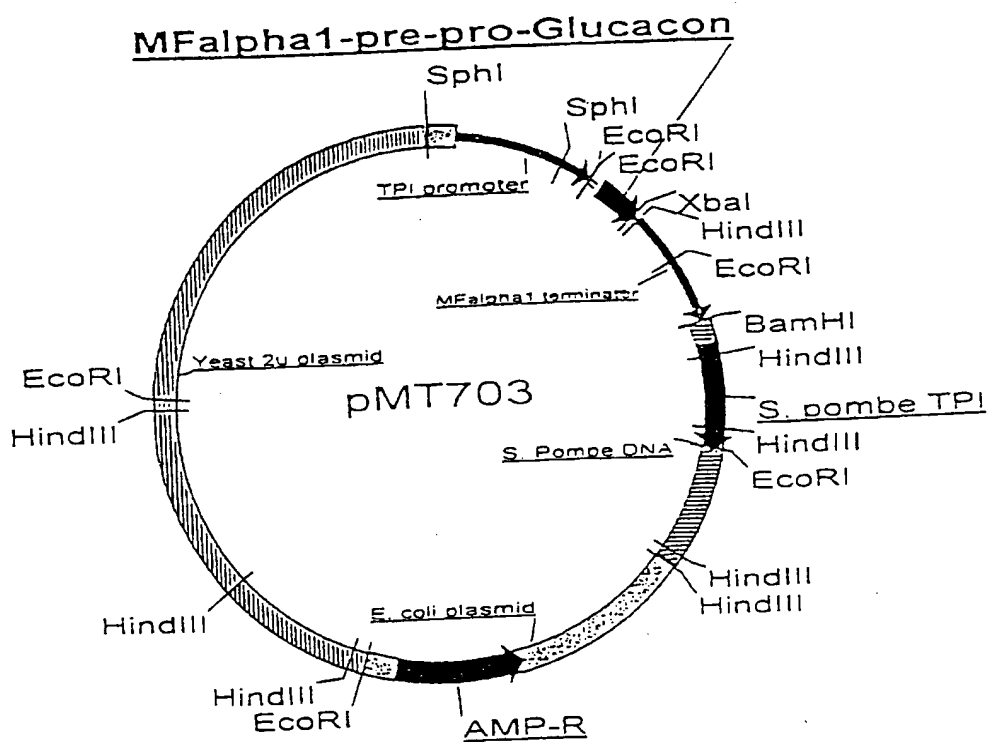


Fig. 3

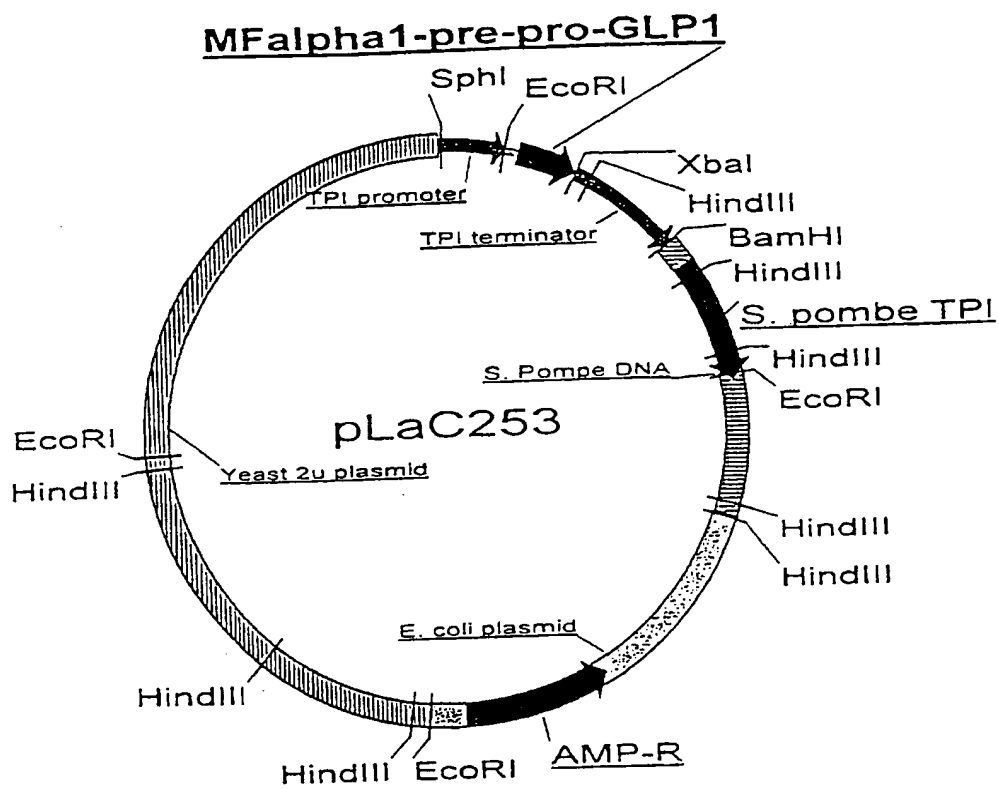


Fig. 4

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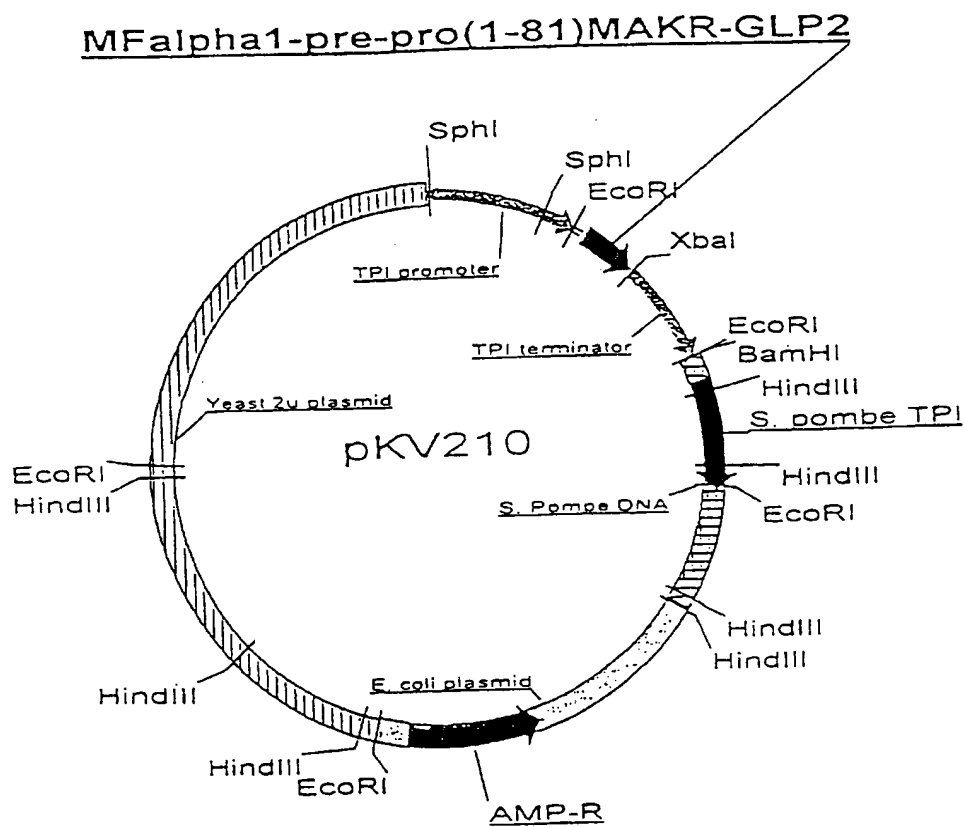


Fig. 5

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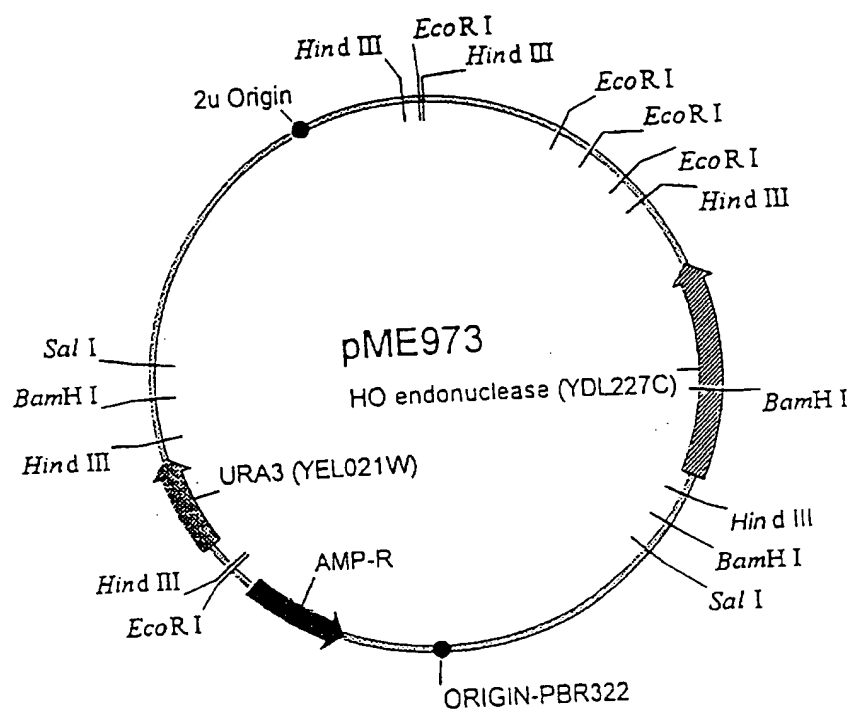


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00298

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 1/19, C07K 14/605 // C12N 15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS DBA, SCISEARCH, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9523857 A1 (DELTA BIOTECHNOLOGY LIMITED), 8 Sept 1995 (08.09.95) --	1-38
A	Dialog Information Service, file 154, Medline, Dialog accession no. 06480997, Medline accession no. 90382674, Gabrielsen OS et al: "Efficient secretion of human parathyroid hormone by Saccharomyces cerevisiae", Gene (NETHERLANDS) Jun 15 1990, 90 (2) p255-62 --	1-38

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

23 October 1997

Date of mailing of the international search report

28 -10- 1997

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00298

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Service, file 154, Medline, Dialog accession no. 06460032, Medline accession no. 90224362, Egel-Mitani M. et al: "A novel aspartyl protease allowing KEX2-independent MF alpha propheremone processing in yeast", Yeast (ENGLAND) Mar-Apr 1990, 6 (2) p127-37</p> <p style="text-align: center;">-- -----</p>	1-38

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/DK 97/00298

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9523857 A1	08/09/95	AU 1818395 A	18/09/95
		CA 2183241 A	08/09/95
		EP 0749478 A	27/12/96
		GB 2301365 A,B	04/12/96
		GB 9404270 D	00/00/00
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